# Human Bone Marrow Stromal Cells have Mitogenic Activity on SK-Hep-1 cells

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Abstract of thesis entitled:

# Human Bone Marrow Stromal Cells have Mitogenic Activity on SK-Hep-1 cells

Submitted by Siu, Yeung Tung for the degree of Master of Philosophy in Surgery at The Chinese University of Hong Kong in Jan, 2001.

The rapid regulation of growth factors including hepatocyte growth factor, interleukin-6 and epidermal growth factor are essential in triggering quiescent hepatocytes to proliferate after liver injury. However, direct infusion of above cytokines only have a little effect on hepatic DNA synthesis indicating that other mitogenic signal is responsible for the initiation of hepatocytes proliferation *in vivo*. It has been shown that hepatocytes can arise from a cell population originating in the bone marrow, and bone marrow stromal cells serve an important role in bone marrow cell proliferation and differentiation. Thus, it was speculated that bone marrow stromal cells may also play a role in regulating hepatocytes proliferation. In this project, SK-Hep-1 cells were selected for the detection of the mitogenic activity of human bone marrow stromal cells from five hepatic cell lines, Hep 3B, Hep G2, C3A, SK-Hep-1 and Chang cells because of its ability to be growth arrested. It was demonstrated that human bone marrow stromal cellular extracts exhibited mitogenic activity on partially growth arrested SK-Hep-1 cells in a dosage dependent manner. The major mitogenic activity of the stromal cellular extract was protein in nature as shown in its sensitivity to heat and proteinase treatment. In conclusion, human bone marrow stromal cells had mitogenic activity on SK-Hep-1 cells.

# 人骨髓基質細胞的促肝細胞分裂活性

2001 年 1 月，香港中文大學外科課程哲學碩士論文摘要

蕭揚東

發生肝損傷時，肝細胞生長因子，白細胞介素-6 及表皮細胞生長因子等生長因子的快速調節在引發肝細胞從靜態到再生中起著重要作用。 但是，直接灌注上述細胞因子僅對肝臟中 DNA 合成產生輕微影響，說明在體內還有其他促細胞分裂因子調節肝細胞的再生。 有證據顯示肝細胞生長可以起源於骨髓，而骨髓基質細胞在骨髓細胞的增殖和分化中擔當重要角色，因此，骨髓基質細胞可能在調節肝細胞再生中起一定作用。 在 Hep 3B, Hep G2, C3A, SK-Hep-1 and Chang 五種肝細胞中，我們的研究表明 SK-Hep-1 細胞最能夠被抑制生長，所以被選作測試的細胞模型。 在生長受到抑制的肝臟 SK-Hep-1 細胞模型中，人骨髓基質細胞的提取物具有促進其分裂的活性。 對提取物加熱或用蛋白酶消化後這種活性大部份消失，說明在提取物中起作用的成份主要是蛋白質。 因此，人骨髓基質細胞具有促進肝臟 SK-Hep-1 細胞分裂的功能。

# Acknowledgement

Dedicate to my Parents and Sister

It is an honor to present you this thesis today. And I am honor to be sailing with the following persons together making such achievement. Once again, we are sailing in the sea of knowledge, the race against ourselves. The thunderstorm does not realize our firmness of purpose and will try everything to test us. But she will test for her own embarrassment while we are exploring for a new land. This reminds me those days when I was having Dr. Mun-fai Leung to demonstrate me how to rig and sail close to the wind at the beginning of voyage. The people before me, Stella Ching-ping Chan, Elut Kwok, Frances Chan, Ivy So, Jie Huang, Ping Xie and Stephen Ho play this game and play it well. Please allow me to express my gratitude to all of them. And a room in my heart dedicates to those friends of mine especially Stella and Dr. Leung for all the great sirloins, guidance and illumination.

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Siu, Yeung Tung

Jan, 2001.

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# Abbreviations

| | |
|---|---|
| 2-AAF | 2-acetylaminofluorene |
| BSA | bovine serum albumin |
| $CCl_4$ | carbon tetrachloride |
| CFU-G | colony-forming units-granulocyte |
| CFU-GM | colony-forming units-granulocyte macrophage |
| CSFR | colony-stimulating factor receptor |
| $_{DL}$-DTT | $_{DL}$-dithiothreitol |
| DMEM | Dulbecco's modified Eagle's medium |
| DMSO | dimethyl sulphoxide |
| EDTA | ethylenediaminetetraacetic acid |
| EGF | epidermal growth factor |
| EGFR | epidermal growth factor receptor |
| FBS | fetal bovine serum |
| FGF | acidic fibroblast growth factor |
| G-CSF | granulocyte colony-stimulating factor |
| G-CSFR | granulocyte colony-stimulating factor receptor |
| GM-CSF | granulocyte-macrophage colony-stimulating factor |
| GM-CSFR | granulocyte-macrophage colony-stimulating factor receptor |
| HBGF-1 | heparin-binding growth factor type I |
| HGF | hepatocyte growth factor |
| HSS | hepatic stimulatory substance |
| IEF | isoelectric focusing |
| IGFBP1 | insulin-like growth factor binding protein-1 |
| IL | interleukin |

| | |
|---|---|
| IL-R | interleukin receptor |
| IPG Strip | immobiline drystrip gel |
| LRF-1 | liver regeneration factor-1 |
| LTBMC | long-term bone marrow culture |
| M-CSF | macrophage colony-stimulating factor |
| MWCO | molecular weight cut-off |
| NF-κB | nuclear factor kappa B |
| PBS | phosphate-buffered saline |
| PHx | partial hepatectomy |
| pro-HGF | single-chain from HGF |
| RNase A | ribonuclease A |
| SCF | stem cell factor |
| SDS | sodium dodecyl sulfate |
| SDS-PAGE | sodium dodecyl sulfate-polyacrylamide gel electrophoresis |
| SF | scatter factor |
| TGF-α | transforming growth factors-α |
| TGF-α R | transforming growth factors-α receptor |
| TGF-β | transforming growth factor-β |
| TGF-βR | transforming growth factor-β receptor |
| TLCK | N-tosyl-L-lysine chloromethyl ketone |
| TNF-α | tumor necrosis factor-α |
| TNF-αR | tumor necrosis factor-α receptor |
| STAT3 | signal transducer and activator of transcription-3 |
| uPA | urokinase |
| uPA-R | urokinase receptor |
| VEGF | vascular endothelial growth factor |

# Chapter 1 Introduction

## 1.1 Growth factors involved in hepatocytes proliferation

Mature hepatocytes are quiescent and highly differentiated liver cells. During liver regeneration, these mature hepatocytes are first to proliferate followed by the growth of other mature liver cell populations including biliary ductular cells, Kupffer cells, Ito cells and sinusoidal endothelial cells after partial hepatectomy (PHx) or hepatic toxins injury. These proliferating hepatocytes produce growth factors leading to the growth of other liver cell populations by paracrine mechanism. The growth factors involved in hepatocytes proliferation during liver regeneration were described (Michalopoulos and DeFrances, 1997). These include hepatocyte growth factor (HGF), tumor necrosis factor-α (TNF-α), interleukin-6 (IL-6), epidermal growth factor (EGF), transforming growth factor-α (TGF-α) and transforming growth factor-β (TGF-β). The entire process of proliferation is precisely regulated and once the proportional liver mass is attained, hepatocytes revert to their growth arrested state.

### 1.1.1 Hepatocyte growth factor (HGF)

The hepatocyte growth factor / scatter factor (SF) and its receptor c-Met (Naldini et al., 1991) are the key factors in early signaling pathway of hepatocyte proliferation. The HGF is the most potent hepatocyte mitogen *in vitro* and *in vivo* (Nakamura et al., 1989). After PHx, dramatically translocation of urokinase receptor (uPA-R) to hepatic plasma membrane within 1 min resulting in enhanced activity of urokinase (uPA) (Mars et al., 1995). The activated uPA converts plasminogen into plasmin,

which in turn activates matrix degrading metalloproteases. The activated metalloproteases participate in proteolysis of hepatic biomatrix, which is a source rich of matrix-bound inactive single-chain from HGF (pro-HGF) (Rifkin, 1992; Blasi, 1993). The released pro-HGF was also activated by uPA into active two-chain form HGF, the high affinity ligand for the c-Met receptor through the extracellular hydrolysis of the Arg494-Val495 (Mars et al., 1993; Naldini et al., 1995). This accounts for the plasma concentrations of HGF rises 17-fold within 2 hr after PHx in rat (Lindroos et al., 1991). Released HGF induces some immediate early genes, including liver regeneration factor-1 (LRF-1) and insulin-like growth factor binding protein-1 (IGFBP1) with a maximum expression 3 hr after PHx (Weir et al., 1994).

### 1.1.2 Tumor necrosis factor-α (TNF-α) and interleukin-6 (IL-6)

Both tumor necrosis factor-α and interleukin-6 are essential in early signaling pathway of hepatocyte proliferation. The plasma concentrations of IL-6 rises 12-fold after PHx in rat (Rai et al., 1996). IL-6 is secreted by Kupffer cells under the stimulation of TNF-α. Signal transducer and activator of transcription-3 (STAT3), a transcription factor for many immediate-early genes, involving in hepatocytes proliferation, can be activated by IL-6. DNA synthesis and STAT3 activity after PHx are severely impaired in mice with TNF-α type I receptor deficiency. Injection of IL-6 before PHx corrects this defect (Yamada et al., 1997). The DNA synthesis of hepatocyte during liver regeneration is suppressed in mice with a homozygous deletion of the IL-6 gene (Cressman et al., 1996). However the mitogenic effect of IL-6 on cultured hepatocytes cannot be determined as both stimulating (Kuma et al., 1990) and inhibiting (Kordula et al., 1991) effect had been reported.

### 1.1.3 Epidermal growth factor (EGF) and transforming growth factor-α (TGF-α)

Epidermal growth factor (EGF) is involving in early signaling pathway of hepatocytes proliferation while transforming growth factors-α (TGF-α) involves at a latter time. TGF-α belongs to the EGF/TGF-α family and shares 35 % sequence homology with EGF and nearly identical spectrum of biological activities with EGF (Massague, 1990). Both EGF and TGF-α bind to the epidermal growth factor receptor (EGFR) on hepatocytes (Massague, 1990). Both EGF and TGF-α are mitogens for cultured hepatocytes. EGF is continually made available to the hepatocytes by salivary glands or Brunner's glands of the duodenum (Skov et al., 1988). There is about 20 % increased in plasma concentrations of EGF after PHx in rat (Noguchi et al., 1991). Removal of salivary gland delays the peak of DNA synthesis of hepatocytes after PHx in rat and such defect can be restored by the administration of EGF (Jones, Jr. et al., 1995). Rapid tyrosine phosphorylation of EGF receptor occurs shortly after PHx suggesting that EGF involved in the early hepatocytes proliferation (Rubin et al., 1982). There is about 9-fold increased in TGF-α mRNA expression in hepatocytes 24 hr after PHx (Mead and Fausto, 1989). But there is only a small rise in plasma concentrations of TGF-α after PHx (Tomiya and Fujiwara, 1996a; Tomiya and Fujiwara, 1996b). This suggests that TGF-α is made available to hepatocytes through autocrine amplification mechanism in a latter stage of hepatocytes proliferation. TGF-α secreted from hepatocytes is also acted as mitogen for the surroundings endothelial cells such as biliary ductular cells. DNA synthesis in those endothelial cells starts 24 hr after PHx (Michalopoulos and DeFrances, 1997). That is the time of TGF-α being expressed by hepatocytes after PHx suggesting that hepatocytes stimulate proliferation of other liver cell

populations by a paracrine mechanism. Acidic fibroblast growth factor/Heparin-binding growth factor type I (FGF/HBGF-1) (Kan et al., 1989) and vascular endothelial growth factor (VEGF) (Mochida et al., 1996) are also secreted by proliferating hepatocytes that can also trigger mitosis of other liver cell populations. Interestingly, the DNA synthesis of hepatocytes during liver regeneration proceeds normally in mice with a homozygous deletion of the TGF-α gene (Russell et al., 1996). This may be compensated by the increase in other ligands in EGF receptor family.

### 1.1.4 Other comitogens

There are other factors, by itself, having no significant proliferative effect on hepatocytes. However these comitogens can synergistically enhance the stimulatory effect of hepatocyte mitogens. They include hormones and neurotransmitters, such as insulin, glucagon, insulin-like growth factors, norepinephrine, thyroid hormones and hepatic stimulatory substance (HSS) (Steer, 1995; Michalopoulos and DeFrances, 1997). However most of their functions and regulatory pathways in hepatocytes proliferation are unknown.

In fact, more than 70 immediate-early genes in hepatocytes have been identified after PHx, 41 of which are novel (Haber et al., 1993). Infusion of HGF, EGF and TGF-α in normal rat directly through the portal vein can only have a little effect on hepatic DNA synthesis (Webber et al., 1994). This indicates that other mitogenic signal is required for hepatocytes to leave quiescent state.

### 1.1.5 Transforming growth factor-β (TGF-β)

After PHx, the regenerating liver attained its original mass, and hepatocytes revert to their growth arrested state and stop growing. Transforming growth factor-β (TGF-β) secreted by Ito cells inhibits DNA synthesis in hepatocytes *in vitro* and *in vivo* (Carr et al., 1986; Russell et al., 1988). The expression of TGF-β receptor on hepatocytes decreases to the lowest level 24 hr after PHx (Chari et al., 1995). While TGF-β mRNA level continues increasing with a plateau at about 48 hr (Braun et al., 1988). The expression of TGF-β receptor recovers to 60 % of pre-PHx level 120 hr after PHx. This down regulation of TGF-β receptor allows the hepatocytes to proliferate while the TGF-β level is increasing. There is no other specific hepatocyte growth inhibitor was known.

Major growth factors that involved in hepatocytes proliferation during liver regeneration were summarized in the table 1 (Michalopoulos and DeFrances, 1997).

Table 1. Growth factors and receptor interactions during liver regeneration

| Growth factor | Source of growth factor | Receptor | Location of receptor | Activity of growth factor /receptor interaction |
|---|---|---|---|---|
| HGF | Hepatic biomatrix | c-Met | Hepatocytes | Mitogenic effect; key factor in immediate early signaling of DNA synthesis |
| TNF-α | Kupffer cells | TNF-αR | Kupffer cells | Stimulate IL-6 secretion from Kupffer cells |
| IL-6 | Kupffer cells | IL-6R | Hepatocytes | Essential in early signaling of DNA synthesis |
| EGF | Salivary glands or Brunner's glands of duodenum | EGFR | Hepatocytes | Mitogenic effect; involved in early signaling of DNA synthesis |
| TGF-α | Hepatocytes | EGFR | Hepatocytes | Mitogenic effect; involved in a latter stage of DNA synthesis |
| TGF-β | Ito cells | TGF-βR | Hepatocytes | Inhibition on DNA synthesis; involved in terminal stage of liver regeneration |

EGF, epidermal growth factor; EGFR, epidermal growth factor receptor; HGF, hepatocyte growth factor; IL-6, interleukin-6; IL-6R, interleukin-6 receptor; TGF-α, transforming growth factor-α; TGF-αR, transforming growth factor-α receptor; TGF-β, transforming growth factor-β; TGF-βR, transforming growth factor-β receptor; TNF-α, tumor necrosis factor-α; TNF-αR, tumor necrosis factor-α receptor

## 1.2 Bone marrow stromal cells and hepatocytes proliferation

### 1.2.1 Role of bone marrow stromal cells in bone marrow

Human bone marrow stromal cells provide a microenvironment to regulate and sustain the self-renewal and differentiation of hematopoietic stem cells. This is achieved through a direct interaction between stromal cells and stem cells mediated by adhesion molecules and growth factors released from the stromal cells (Dorshkind, 1990; Quesenberry, 1992). In long-term bone marrow culture (LTBMC), those stem cells and immature precursors adhere tightly to the stroma while those more mature precursors and differentiated cells are found as nonadherent cells (Coulombel et al., 1983; Harrison et al., 1987; Kierney and Dorshkind, 1987). This suggests that the decomposition of these adhesion molecules, collagen, fibronectin and laminin of the extracellular biomatrix (Gallagher et al., 1983; Zuckerman and Wicha, 1983; Campbell et al., 1985) and the release of the matrix-bound stromal-derived factors, like granulocyte-macrophage colony-stimulating factor (GM-CSF) (Gordon et al., 1987; Roberts et al., 1988) are necessary for hematopoiesis. Numerous stromal-derived factors that can facilitate the proliferation and differentiation of hematopoietic cells are identified (Ogawa, 1993). These factors can be characterized into myelopoietic, erythropoietic and lymphopoietic factors with respect to their effect on lineage specific hematopoietic cell growth and differentiation. There are at least 12 lymphopoiesis regulatory stromal-derived cytokines produced during inflammatory episodes suggesting that stromal cells can response to injury by changing its pattern of cytokines production (Dainiak, 1991; Kincade, 1991).

### 1.2.2 Bone marrow as a source of hepatic oval cells

When the mature hepatocytes are prevented from proliferation after PHx by exposure to 2-acetylaminofluorene (2-AAF), hepatic oval cells will proliferate and differentiate to generate new hepatocytes instead (Fausto et al., 1993; Sell, 1994; Thorgeirsson, 1996). Compared the phenotype of bone marrow stem cells and oval cells, they both express CD34 (Omori et al., 1997a), Thy-1 (Petersen et al., 1998), c-kit (Fujio et al., 1994) and flt-3 receptor mRNA (Omori et al., 1997b). The cell lineage capacity of bone marrow in hepatic oval cells or hepatocytes was investigated (Petersen et al., 1999). Cross-sex bone marrow transplantation was used to trace the origin of the liver cells. Recipient female rat was also treated with 2-AFF to block hepatocytes proliferation and then $CCl_4$, a sort of hepatic toxin, to induce oval cells proliferation and differentiation. It had demonstrated that Y chromosome-positive oval cells and hepatocytes present in liver of lethally irradiated syngeneic female rat with bone marrow transplantation from a male rat, after 2-AAF-$CCl_4$ treatment by day 9 and day 13. If all oval cells that differentiate into hepatocytes were derived from the female recipient liver, one would expect that none of the oval cells or hepatocytes tested would be positive for Y chromosome. Moreover, the time lag between day 9 and day 13 was corresponding to the time that is required for oval cells to differentiate into hepatocytes. This suggests that the oval cells can arise from a cell population originating in the donor bone marrow and continued to differentiate into hepatocytes by day 13. Since one of the role of bone marrow stromal cells is to regulate the proliferation of bone marrow originated cells. We suspect that stromal cells can also play a role in regulating hepatocytes proliferation.

### 1.2.3 Growth factors secreted by bone marrow stromal cells involved in hepatocytes proliferation

Secretion of cytokines may be a way to regulate hepatocytes proliferation by bone marrow stromal cells. In addition to GM-CSF, macrophage colony-stimulating factor (M-CSF), granulocyte colony-stimulating factor (G-CSF), IL-1β, IL-7, IL-11, and stem cell factor (SCF), stromal-derived factors also include HGF, TGF-β, TNF-α and IL-6 which are essential factors in hepatocytes proliferation as discussed above. HGF, initially identified as a potent mitogen for hepatocytes, was shown to promote myelopoiesis through stimulating colony-forming units-granulocyte macrophage (CFU-GM) formation in LTBMC (Kmiecik et al., 1992; Mizuno et al., 1993; Galimi et al., 1994; Nishino et al., 1995; Takai et al., 1997). Myelopoiesis can be down regulated by TGF-β (Cashman et al., 1990; Takai et al., 1997), which is also a growth inhibitor in hepatocytes. Both stromal cells and Kupffer cells can be triggered by TNF-α to release IL-6 (Khoury et al., 1992; Rougier et al., 1998). IL-6 promotes myelopoiesis through stimulating colony formation from colony-forming units-granulocyte (CFU-G) and macrophage colony formation in LTBMC (Bot et al., 1989; Liu et al., 1997). And IL-6 is also essential in early signaling pathway of hepatocytes proliferation. Some of the stromal-derived factors were shown to be essential in triggering quiescent hepatocytes to proliferate after liver injury. Therefore the potential mitogenic activity of human bone marrow stromal cells on hepatocytes is expected.

The growth factors that are constitutively produced from unstimulated bone marrow stromal cells cultivating with serum were summarized in table 2 (Dorshkind, 1990; Nagao, 1995; Takai et al., 1997).

Table 2. The constitutively produced bone marrow stromal-derived factors with their major effects on different lineages of hematopoietic cells

| Lineage specificity | Growth factor | Receptor | Target cells | Activity of growth factor /receptor interaction |
|---|---|---|---|---|
| Myelopoietic factors | G-CSF | G-CSFR | CFU-G | Stimulate proliferation and differentiation of CFU-G |
| | M-CSF | CSFR | CFU-M, Monocytes/ Macrophages | Stimulate proliferation and differentiation of CFU-M, activation of monocytes/ macrophages |
| | GM-CSF | GM-CSFR | CFU-GM, Neutrophils, CFU-Mk | Stimulate proliferation and differentiation of CFU-GM, CFU-Mk, activation of neutrophils |
| | **HGF*** | c-Met | CFU-GM | Stimulate proliferation and differentiation of CFU-GM |
| | IL-1β | IL-1R | Macrophages | Activation of macrophages |
| | IL-3 | IL-3R | CFU-GM, CFU-Mk | Stimulate proliferation and differentiation of CFU-GM, CFU-Mk |
| | **IL-6*** | IL-6R | CFU-GM, CFU-M, CFU-Mk | Stimulate proliferation and differentiation of CFU-GM, CFU-M, CFU-Mk |
| | IL-7 | IL-7R | Monocytes | Stimulate cytokines production from monocytes |
| | IL-11 | IL-11R | CFU-Mk | Stimulatie proliferation and differentiation of CFU-Mk |
| | SCF | KIT | CFU-GM, CFU-Mk | Stimulate proliferation and differentiation of CFU-GM, CFU-Mk |
| | **TGF-β*** | TGF-βR | CFU-GM | Inhibition on proliferation and differentiation of CFU-GM |
| Erythropoietic factors | GM-CSF | GM-CSFR | BFU-E | Stimulate proliferation and differentiation of BFU-E |
| | IL-3 | IL-3R | BFU-E | Stimulate proliferation and differentiation of BFU-E |
| | IL-11 | IL-11R | BFU-E | Stimulate proliferation and differentiation of BFU-E |
| | SCF | KIT | BFU-E | Stimulate proliferation and differentiation of BFU-E |

| | | | | |
|---|---|---|---|---|
| | IL-3 | IL-3R | Pre-B cells | Stimulate proliferation and differentiation of Pre-B cells |
| Lymphopoietic factors | IL-7 | IL-7R | Pre-B cells, cortical thymocyte, T-cell | Stimulate proliferation and differentiation of Pre-B cells, cortical thymocyte, T-cell |

* These stromal-derived factors were also shown to be essential in hepatocytes proliferation after liver injury as discussed in section 1.1 and summarized in table 1.

G-CSF, granulocyte colony-stimulating factor; G-CSFR, granulocyte colony-stimulating factor receptor; M-CSF, macrophage colony-stimulating factor; CSFR, colony-stimulating factor receptor; GM-CSF, granulocyte-macrophage colony-stimulating factor; GM-CSFR, granulocyte-macrophage colony-stimulating factor receptor; HGF, hepatocyte growth factor; IL, interleukin; ILR, interleukin receptor; SCF, stem cell factor; TGF-β, transforming growth factor-β; TGF-βR, transforming growth factor-β receptor

### 1.2.4 Endocrine in hepatocytes proliferation

When the parabiotic circulation of two rats are connected surgically, PHx in one unit can trigger hepatocytes proliferation in both units (Moolten and Bucher, 1967; Fisher et al., 1971). In addition, after PHx, the first portion of hepatocytes in hepatic lobules undergo proliferation was periportal zone (Rabes et al., 1976) prolonging to the pericentral zone. Periportal zone surrounds portal triads which includes portal vein, hepatic artery and bile ductule. The inactive, hepatic biomatrix-bound HGF is retained primarily at the periportal zone (Liu et al., 1994). Hepatic uptake of EGF in rat after an intravenous injection is also retained primarily at the periportal biomatrix (St Hilaire et al., 1983). Hepatocytes contain significant intracellular concentrations of TGF-β 12 hr after PHx. The increase of internalized TGF-β concentration is starting from periportal zone prolonging to the pericentral zone (Jirtle et al., 1991). The hepatic oval cells also originate from the cells present in the canals of Herring, the smallest intralobular biliary ducts or small marginal interlobular biliary ducts located at the periportal zone and from blast-like cells located next to the bile ducts (Novikoff et al., 1996). These evidences suggested that blood circulation delivered mitogen(s), which could trigger the liver regeneration after PHx starting from the area closest to hepatic artery. The highly localized biomatrix-bound pro-HGF stock, EGF uptaken capacity of biomatrix and the removal of TGF-β inhibition from the extracellular environment of hepatocytes reinforce the importance of periportal zone, the hepatocytes proliferation ready area in response to blood delivering mitogens. However, the function of hepatic oval cells on regulating hepatocytes proliferation need to be clarified. In conclusion, it is reasonable to postulate that bone marrow stromal cells may regulate the hepatocytes proliferation through its growth factors secretion by an endocrine mechansim.

## 1.3 Objective of this study

The objective of this study was to demonstrate that the human bone marrow stromal cells have mitogenic activity on hepatocytes by (i) selection of human hepatic cell line for the detection of mitogenic activity; (ii) detection of mitogenic activity of human bone marrow stromal cells on the selected cell line, SK-Hep-1 cells; (iii) characterization of hepatocyte mitogenic activity of bone marrow stromal cellular extract; and (iv) performing a preliminary test on the difference between bone marrow stromal cellular extract and other growth factors.

(i) "Selection of human hepatic cell line for the detection of mitogenic activity" was determined by the ability of human hepatic cell lines, Hep 3B, Hep G2, C3A, SK-Hep-1 and Chang cells to be enriched at G0-G1 phases of the cell cycle by serum deprivation (Campisi et al., 1984). Cell cycle distribution changes of serum deprived hepatic cells with or without mitogenic stimulation were also determined to ensure that blocking cells from cycling was not result in death of the cells.

The DNA synthesis of human hepatocarcinoma, Hep 3B, Hep G2, C3A, SK-Hep-1 and Chang cells can be triggered by hepatocyte mitogens, HGF, EGF and TGF-$\alpha$ (Lee et al., 1998; Guren et al., 1999; Tamura and Daikuhara, 2000). Hep 3B cells have been shown to be an experimental model for investigation of plasma protein biosynthesis of liver (Knowles et al., 1980). Hep G2 and SK-Hep-1 cells have been shown to be a well-characterized model for studying liver detoxification including cytochrome P450 activity (Gabelova et al., 2000). C3A cells are clonal derivative of Hep G2 cells that are selected for strong contact inhibition of growth and high

albumin production. Chang cells are derived from normal liver tissue. Selection was made from these hepatic cell lines.

Serum deprivation for obtaining G0-G1 phases has been used in many cell cycle-related studies, including analysis of the cell cycle-dependent reversible tyrosine phosphorylation of cdc2 (Morla et al., 1989) and regulation of cyclin A (Carbonaro-Hall et al., 1993). Cells synchronized at the G1-S boundary by chemical inhibitor, thymidine, mimosine, or aphidicolin had 4 fold higher levels of cyclins E and B1 suggesting that chemical treatment has been linked to the possible disruption of normal cell cycle regulatory process (Schimke et al., 1991; Gong et al., 1995). Thus serum deprivation was used for obtaining enrichment of G0-G1 phases in this project.

(ii) "Detection of mitogenic activity of human bone marrow stromal cells on the selected cell line, SK-Hep-1 cells" was defined by cell cycle distribution changes of partially growth arrested SK-Hep-1 cells before and after primary bone marrow stromal cellular extract incubation. The primary stromal cells were obtained from fresh human bone marrow biopsy sample of leukemia patient.

All of the stromal cell lines being reported have different predominant cytokines production indicating that stromal cell lines are lineage restricted in its hematopoietic support capacity (Greenberger, 1991). It is well established that pluripotent stem cells and other precursors in different hematopoietic lineages including pro-B-cells, cortical thymocyte, CFU-GM, CFU-G, CFU-M, CFU-E and CFU-Mk can persist for several weeks in LTBMC as cultivating with primary stromal cells(Muller-Sieburg and Deryugina, 1995). Thus primary stromal culture was used in order to exclude the predominant cytokines production of cell lines-derived cytokines.

The primary stromal cells were obtained from human bone marrow according to two well-established culture systems for LTBMC (Dexter et al., 1977; Whitlock and Witte, 1982). Briefly, stromal cells were cultured in medium supplemented with relatively high concentration of fetal calf serum, usually 15 - 25 %. Thus co-culturing of partially growth arrested SK-Hep-1 cells and stromal cells was unable to determine the mitogenic activity of stromal cells because the constitutively production of cytokines from stromal cells required under cultivating with high concentration of serum, which was already a strong mitogens for serum deprived SK-Hep-1 cells. The alternative, stromal cellular extract was used for the detection of mitogenic activity of stromal cells on SK-Hep-1 cells. The preparation of stromal cellular extract was prepared as described by Ho and his colleagues (Ho et al., 1998).

Leukemia defines as fatal diseases of the reticuloendothelial system involving uncontrolled proliferation of leukocytes. The primary stromal cells were obtained from fresh human bone marrow biopsy sample of leukemia patient because of the availability of bone marrow sample. The bone marrow stromal cells obtained were presumably normal (Ho et al., 1998).

(iii) "Characterization of hepatocyte mitogenic activity of bone marrow stromal cellular extract" was defined by dialysis, temperature treatment, and proteolysis.

(iv) "Performing a preliminary test on the difference between bone marrow stromal cellular extract and other growth factors" was determined by the comparison of mitogenic response and early intracellular signaling of SK-Hep-1 cells in response to bone marrow stromal cellular extract and other growth factors. These experiments provided a basis evidence for detail experimental study.

# Chapter 2 Materials and Methods

## 2.1 Cell cultures

Human hepatocytes, Hep 3B and SK-Hep-1 (American Type Culture Collection, Manassas, VA, USA) were routinely maintained in Dulbecco's modified Eagle's medium (DMEM, GIBCO-BRL, Life Technologies, Gaithersberg, MD, USA) supplemented with 10 % fetal bovine serum (FBS, GIBCO), while Hep G2, C3A and Chang cells (ATCC) were routinely maintained in Minimum essential medium (MEM, GIBCO) supplemented with 10 % FBS. Primary human bone marrow stromal cells were routinely maintained in DMEM supplemented with 15 % FBS. All cell cultures were incubated in humidified air with 5 % $CO_2$ at 37 $^0$C by $CO_2$ water jacketed incubator (Forma Scientific Inc., Marietta, OH, USA). Cell count was determined via Coulter Particle Count and Size Analyser (Coulter electronics Ltd., Northwell Drive, Luton, England). Cell doubling time was calculated as $N = N_0 \times 2^{(t/td)}$ where N equals to final cell count, $N_0$ equals to seeding cell count, t equals to the culturing time and td equals to the cell doubling time. All cells were frozen with 67% culture medium, 25% FBS and 8% dimethyl sulphoxide (DMSO, Sigma, St. Louis, MO, USA).

## 2.2 Selection of human hepatic cell line for the detection of mitogenic activity

### 2.2.1 Enrichment of human hepatic cell lines, Hep 3B, Hep G2, C3A, SK-Hep-1 and Chang cells at G0-G1 phases by serum deprivation

Enrichment of hepatic cell lines, Hep 3B, Hep G2, C3A, SK-Hep-1 and Chang cells at G0-G1 phases were obtained by serum deprivation. Hep 3B and SK-Hep-1 cells were plated at 3,200 cells/cm$^2$ (0.015 x 10$^6$ cells/ml x 6 ml in 60 mm dish) in DMEM supplemented with 10 % FBS for 24 hr, while Hep G2, C3A and Chang cells were plated at 3,200 cells/cm$^2$ in MEM supplemented with 10 % FBS for 24 hr. Then the cells were washed once with culture medium followed by incubating in culture medium supplemented with 0.5, 0.3, and 0.1 % FBS. Cells were starved for another 72 hr before experiments.

### 2.2.2 Incubation of serum deprived Hep 3B, Hep G2, C3A, SK-Hep-1 and Chang cells with mitogenic stimuli

The serum deprived hepatic cell lines, Hep 3B, Hep G2, C3A, SK-Hep-1 and Chang cells were incubated with 10 % FBS for 21hr.

### 2.2.3 Cell cycle analysis by flow cytometry using propidium iodide staining

Cell cycle distribution of serum deprived Hep 3B, Hep G2, C3A, SK-Hep-1 and Chang cells with or without mitogenic stimulation were analyzed by propidium iodide staining. Cells were dislodged by trypsinization. Then, the cells were washed once

with 1 ml cold phosphate-buffered saline (PBS, 137 mM sodium chloride, 2.7 mM potassium chloride, 4.3 mM sodium phosphate, dibasic and 1.4 mM potassium phosphare, monobasic, pH 7.4) before fixed with 1 ml cold 95 % ethanol in PBS for 15 minutes. Cells were incubated with 0.1 % ribonuclease A (RNase A, Sigma) at 37 $^{0}$C for 20 min in 500 μl PBS after filtering against 35 μm stainer cap (Falcon, Becton Dickinson, San Jose, CA, USA). Cells were stained with 50 μg/ml propidium iodide solution on ice in dark for 30 min. DNA content of 10,000 events (cells) were gated per sample by flow cytometry (Becton Dickinson) with excitation wavelength 488 nm and emission wavelength 575 nm. Cell cycle distribution was analyzed by ModFit LT program (Verity Software House Inc., Topsham, ME, USA).

## 2.3 Detection of mitogenic activity of human bone marrow stromal cells on the selected cell line, SK-Hep-1 cells

### 2.3.1 Partially growth arrested human SK-Hep-1 cells

The partially growth arrested SK-Hep-1 cells were obtained by the method of serum deprivation. Cells were plated at 3,200 cells/cm$^2$ (0.015 x 10$^6$ cells/ml x 6 ml in 60 mm dish) in DMEM supplemented with 10 % FBS for 24 hr. Then the cells were washed once with DMEM followed by incubating in DMEM supplemented with 0.1 % FBS. Cells were incubated for another 72 hr before experiments.

Limited passages of SK-Hep-1 cells between 8 and 11 were used for experiments in order to minimize clones deviation upon passing. And the cell doubling time should be around 1.3 day before experiments.

### 2.3.2 Human bone marrow stromal cells

Primary human bone marrow stromal cells were obtained from fresh human bone marrow biopsy sample of leukemia patient. Sample was incubated in RPMI medium 1640 (GIBCO) supplemented with 10 % FBS and 100 units/ml penicillin and 100 mg/ml streptomycin (Sigma) for 24 hr. Then suspended hematopoietic cells from the attached stromal cells were washed with DMEM five times followed by growing the cells in DMEM supplemented with 15 % FBS.

#### 2.3.2.1 Bone marrow stromal cellular extract

Bone marrow stromal cells were harvested by trypsinization. Each million cells were washed with 0.5 ml cold phosphate-buffered saline (PBS, 137 mM sodium chloride, 2.7 mM potassium chloride, 4.3 mM sodium phosphate, dibasic and 1.4 mM potassium phosphate, monobasic, pH 7.4) five times. Five millions cells were resuspended in one ml of PBS. Cells were lysed with Ultrasonic Processor (Cole-Parmer Instrument Co., Vernon Hills, IL, USA) with power 2, total process time 30 sec, pulse on time 1 sec and pulse off time 1 sec on ice. Debris was spun down by ultracentrifuge (Beckman Instruments, Inc., Fullerton, CA, USA) using 70.1Ti rotor (Beckman) at 100,000 g at 4 $^{0}$C for 1 hr. The supernatant, the total cellular extract, was further sterilized by filtration using a 0.22 μm filter (USA Scientific, Inc., Ocala, FL, USA). Cell lysate was aliquot and store at -80 $^{0}$C before use.

#### 2.3.2.2 Total protein assay

The total protein concentration of stromal cellular extract was determined by Bradford assay (Bradford, 1976) using bovine serum albumin (BSA, Sigma) as standard.

Protein samples were mixed with 5 X dye reagent concentrate (Bio-Rad Laboratories, Hercules, CA, USA) for 5 min at room temperature. The absorbance was measured at OD 595 nm at room temperature with a spectrophotometer (Beckman). The protein concentration was calculated from the standard curve.

### 2.3.3 Incubation of SK-Hep-1 cells with bone marrow stromal cellular extracts

For the time course experiment on DNA synthesis of SK-Hep-1 cells in response to FBS and bone marrow stromal cellular extract, partially growth arrested SK-Hep-1 cells were incubated with 10 % FBS and 30 μg/ml stromal cellular extract of patient 1 for 6, 9, 12, 15, 18, 21, 24 hr and 15, 18, 21, 24, 25, 27, 30 hr, respectively.

For the dose response experiment on DNA synthesis of SK-Hep-1 cells in response to bone marrow stromal cellular extracts, partially growth arrested SK-Hep-1 cells were incubated with 5, 10, 20, 30 and 40 μg/ml stromal cellular extracts originated from four different patients for 21 hr.

Cell cycle distribution of stromal cellular extract treated SK-Hep-1 cells were analyzed as described in section 2.2.3

## 2.4 Characterization of hepatocyte mitogenic activity of bone marrow stromal cellular extract

### 2.4.1 Dialysis

The bone marrow stromal cellular extract of patient 1 was dialyzed against 2 L PBS with 3 changes of buffer at 4 $^{0}$C for 17 hr. The molecular weight cut-off (MWCO) of dialysis tubings using were 3.5 and 14 kDa (Spectrum Laboratories Inc., Rancho Dominguez, CA, USA). The tubing was first boiled in sodium bicarbonate / EDTA solution (1 mM EDTA and 2 % sodium bicarbonate) for 10 min followed by boiling in 0.1 M EDTA solution for another 10 min. Boiled tubing was rinsed extensively with PBS before use. The dialyzed extract of patient 1 was further sterilized by filtering against 0.22 μm filter and measured the total protein content (section 2.3.2.2) before testing for hepatocyte mitogenic activity as described in section 2.2.3. The partially growth arrested SK-Hep-1 cells was incubated with 15 μg/ml dialyzed stromal cellular extract of patient 1 for 21 hr.

### 2.4.2 Temperature treatment

The bone marrow stromal cellular extract of patient 1 was subjected to a serial temperature treatment, at 22, 37, 45, 65 and 100 $^{0}$C in water bath for 30 min before testing for hepatocyte mitogenic activity as described in section 2.2.3. The partially growth arrested SK-Hep-1 cells was incubated with 30 μg/ml temperature treated stromal cellular extract of patient 1 for 21 hr.

### 2.4.3 Proteolysis

The bone marrow stromal cellular extract of patient 1 was separately digested with 0.3 μg/ml trypsin (Roche Molecular Biochemical, Boehringer Mannheim, Sandhofer Strasse, Mannheim, Germany) and 0.3 μg/ml endoproteinase Lys-C (Roche) at 37 $^{0}$C for 30 min. Proteolysis was inhibited by 10 μg/ml N-Tosyl-L-lysine chloromethyl ketone (TLCK) (Roche) before testing for hepatocyte mitogenic activity as described in section 2.2.3. The partially growth arrested SK-Hep-1 cells was incubated with 30 μg/ml digested stromal cellular extract of patient 1 for 21 hr.

## 2.5 Performing a preliminary test on the difference between bone marrow stromal cellular extract and other growth factors

### 2.5.1 Incubation of SK-Hep-1 cells with bone marrow stromal cellular extract or other growth factors

Partially growth arrested SK-Hep-1 cells were incubated with 30 μg/ml bone marrow stromal cellular extracts of patient 1, 10 % FBS, human recombinant stimulatory growth factors, 30 ng/ml HGF, 2 μg/ml insulin, 1 ng/ml EGF and 1 ng/ml TGFα (Calbiochem – Novabiochem Corporation, La Jolla, CA, USA) and 40 μg/ml cells lysate of itself, for 21 hr. Hepatocyte mitogenic activities were tested as described in section 2.2.3. The preparation of SK-Hep-1 cells lysate was as same as the preparation of stromal cellular extract described in section 2.3.2.1.

### 2.5.2 Metabolic labeling of SK-Hep-1 cells with [$^{32}$P]orthophosphate

Partially growth arrested SK-Hep-1 cells were washed once with 3 ml warm labeling medium, phosphate free DMEM (GIBCO). Then incubate cells with $^{32}P_i$, 0.1 mCi/ml, carrier free (Amersham Pharmacia Biotech UK Ltd., Little Chalfont, Buckinghamshire, England) for 3 hr in 2 ml labeling medium.

### 2.5.3 Incubation of labeled SK-Hep-1 cells with bone marrow stromal cellular extract or other growth factors

After 3 hr labeling, SK-Hep-1 cells were further incubated with 30 μg/ml bone marrow stromal cellular extracts of patient 1, 10 % FBS, 30 ng/ml HGF, 2 μg/ml insulin, 1 ng/ml EGF and 1 ng/ml TGFα for 15, 30, 45 and 60 min.

### 2.5.4 SK-Hep-1 cells lysate extraction

The protein phosphorylation was stopped by washing twice with 20 mM cold Tris-HCl buffer. SK-Hep-1 cells were lysed with 200 μl lysis solution (8 M urea, 4 % triton X-100, 40 mM trizma base and 20 mM DL-dithiothreitol) and further incubated on ice for 20 min. Debris was spun down by microcentrifuge (Eppendorf, Barkhausenweg, Hamburg, Germany) using FA 45-30-11 rotor (Eppendorf) at 10,000 g at 4 $^0$C for 10 min. About 0.035 x $10^6$ cells' lysate was mixed with 200 μl cold rehydration stock solution with IPG buffer (8 M urea, 2 % triton X-100, 0.5 % IPG buffer pH 3-10 linear, trace amount of bromophenol blue and 0.3 % DL-dithiothreitol). Molecular weight and isoelectric point were determined by two-dimensional SDS-PAGE standards (Bio-Rad) seperated on parallel processed gel.

### 2.5.5 Two-dimensional electrophoresis

Two-dimensional electrophoresis was carried out as described by Görg and her colleagues (Görg et al., 1985; Görg et al., 1988) using isoelectric focusing (IEF) in the first dimension and sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) in the second dimension.

#### 2.5.5.1 First dimension isoelectric focusing

Immobiline DryStrip gel, pH 3 – 10 linear, 11 cm (IPG Strip) (Pharmacia) was rehydrated with the sample mixture overnight at room temperature covered with IPG Cover Fluid (Pharmacia) in a reservoir slot of Immobiline DryStrip Reswelling Tray (Pharmacia).

Proteins on IPG strip were focused by Multiphor II unit (Pharmacia) with Immobiline DryStrip Kit (Pharmacia). A total 12,000 V-hr was applied with several gradients increased in voltage up to 3,500 V according to the following conditions:

| Phase | Voltage(V) | Current(mA) | Power(W) | Volt-hr(Vh) | Time(h:min) |
|---|---|---|---|---|---|
| 1 | 300 | 2 | 5 | 1 | 0:01 |
| 2 | 3,500 | 2 | 5 | 2,900 | 1:30 |
| 3 | 3,500 | 2 | 5 | 9,100 | 2:35 |
| | | | Total: | 12,000 | 4:05 |

* Set the temperature on Multi Temp III Thermostatic Circulator (Pharmacia) to $20^0$C. Program power supply (Pharmacia) in gradient mode.

### 2.5.5.2 Second dimension sodium dodecyl sulfate-polyacrylamide gel electrophoresis

Each focused IPG strip was immediately equilibrated with 10 ml SDS equilibration buffer with DTT (0.1 M tris-HCl, 6 M urea, 30 % glycerol, 1 % SDS and 0.5 % DL-dithiothreitol) for 10 min under gentle agitation. The buffer was replaced with 10 ml SDS equilibration buffer with iodoacetamide (0.1 M tris-HCl, 6 M urea, 30 % glycerol, 1 % SDS, trace amount of bromophenol blue and 4.5 % iodoacetamide) for another 10 min under gentle agitation.

Focused proteins were resolved on precast gradient gel, ExcelGel SDS, 5 % stacking gel, 8 – 18 % separating gel (Pharmacia) using Multiphor II unit. A total 1,050 V-hr was applied with several stepwise increased in current up to 50 mA per gel according to the following conditions:

| Phase | Voltage(V) | Current(mA)* | Power(W)* | Volt-hr(Vh) | Time(h:min) |
|---|---|---|---|---|---|
| 1 | 600 | 20 | 30 | 300 | 0:30 |
| 2 | 600 | 50 | 30 | 50 | 0:05 |
| 3 | 600 | 50 | 30 | 700 | 1:10 |
| | | | Total: | 1,050 | 1:45 |

* Set the temperature to 15 $^{0}$C. Program power supply in step mode. Multiple the current and power by 2 for dual gels running simultaneously.

### 2.5.6 Amplification of radiolabeled signal by EN$^{3}$HANCE

The resolving gel was fixed with fixer solution (10 % acetic acid and 40 % ethanol) for 20 min under gentle agitation. After fixation, impregnate the fixed gel in a

fluorescent intermediate, EN$^3$HANCE (NEN$^{TM}$ Life Science Products, Boston, MA, USA) for 1 hr under gentle agitation. Following impregnation, precipitate the fluorescent material inside the gel with Milli Q water (Millipore Corporation, Bedford, MA, USA) for 10 min under gentle agitation.

### 2.5.7 Visualization of autoradiography

The impregnated gel was dried at 70 $^0$C under vacuum for 30 min by Gel Dryer (Bio-Rad) and exposed to BioMax MR autoradiography film (Eastman Kodak Company, Rochester, NY, USA) at –80 $^0$C overnight.

### 2.5.8 Visualization by silver staining

The 2-D SDS-PAGE standards were visualized by using PlusOne Silver Staining Kit, Protein (Pharmacia) with Hoefer Automated Gel Stainer (Pharmacia). Briefly, the resolving gel was fixed in fixer solution for 30 min. The proteins were then rendered more reactive towards silver in sensitizing solution (6.8 % sodium acetate, 0.2 % sodium thiosulfate, 30 % ethanol and 0.125 % glutardialdehyde) for 30 min. After Milli Q washing, the gel was impregnated with silver solution (0.25 % silver nitrate and 0.0148 % formaldehyde) for 20 min. The silver ion was reduced to metallic silver in developing solution (2.5 % sodium carbonate and 0.0074 % formaldehyde) for 4 to 10 min. When the protein spots developed, the gel was incubated with stop solution (1.46 % EDTA) for 10 min preventing from further reduction of silver ion. Finally, the gel was preserved with the 8.7 % glycerol solution. Stained gel was dried as described in section 2.5.7.

# Chapter 3 Results

## 3.1 Selection of human hepatic cell line for the detection of mitogenic activity

### 3.1.1 Enrichment of human hepatic cell lines, Hep 3B, Hep G2, C3A, SK-Hep-1 and Chang cells at G0-G1 phases by serum deprivation

The selection of human hepatic cell line for the detection of mitogenic activity was determined by the ability of hepatic cell lines, Hep 3B, Hep G2, C3A, SK-Hep-1 and Chang cells to be enriched at G0-G1 phases. Only SK-Hep-1 and Chang cells can be synchronized at G0-G1 phases by serum deprivation (Figure 1). There were about 17 % and 14 % rise in G0-G1 phases of the total cell population in SK-Hep-1 and Chang cells as starving with 0.1 % FBS, respectively.

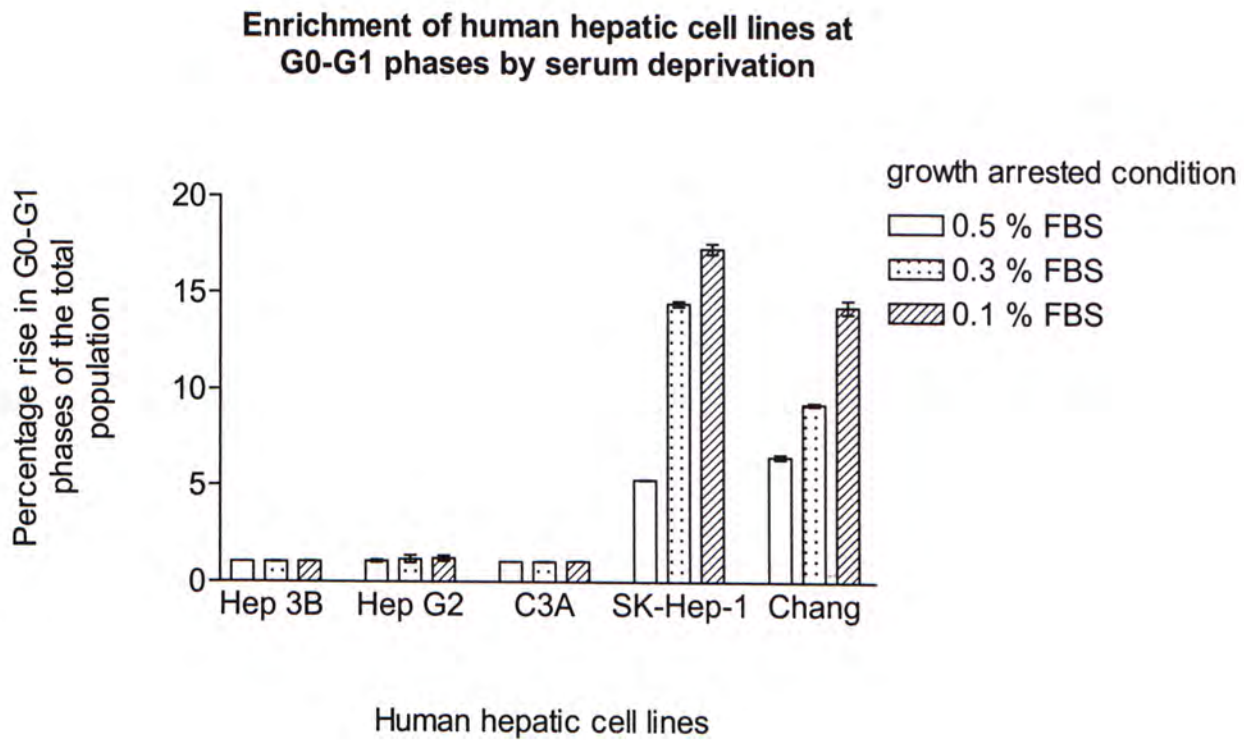



Figure 1. Enrichment of human hepatic cell lines at G0-G1 phases by serum deprivation. Hep 3B, Hep G2, C3A, SK-Hep-1 and Chang cells were seeded in culture medium supplemented with 10 % FBS for 24 hr. Then the cells were washed once with culture medium followed by incubating in culture medium supplemented with 0.5, 0.3, and 0.1 % FBS. Cell cycle analyisis was performed after serum deprivation for 72 hr. "Percentage rise in G0-G1 phases of the total population" was determined by the percentage of cells in G0-G1 phases with serum deprivation treatment minus the percentage of control cells in G0-G1 phases without serum deprivation treatment. Data were mean of two experiments in duplicates $\pm$ S.D.

### 3.1.2 DNA synthesis of hepatic cell lines in response to 10 % FBS after serum deprivation

The DNA synthesis of hepatic cell lines, Hep 3B, Hep G2, C3A, SK-Hep-1 and Chang cells in response to 10 % FBS after previously serum deprivation were determined by cell cycle analysis. Only growth arrested SK-Hep-1 and Chang cells demonstrated the ability to response to mitogenic stimulation after serum deprivation (Figure 2). There were about 31 % and 19 % rise in S/G2-M phases of the total cell population in SK-Hep-1 and Chang cells after 10 % FBS stimulation in cells starving with 0.1 % FBS, respectively.

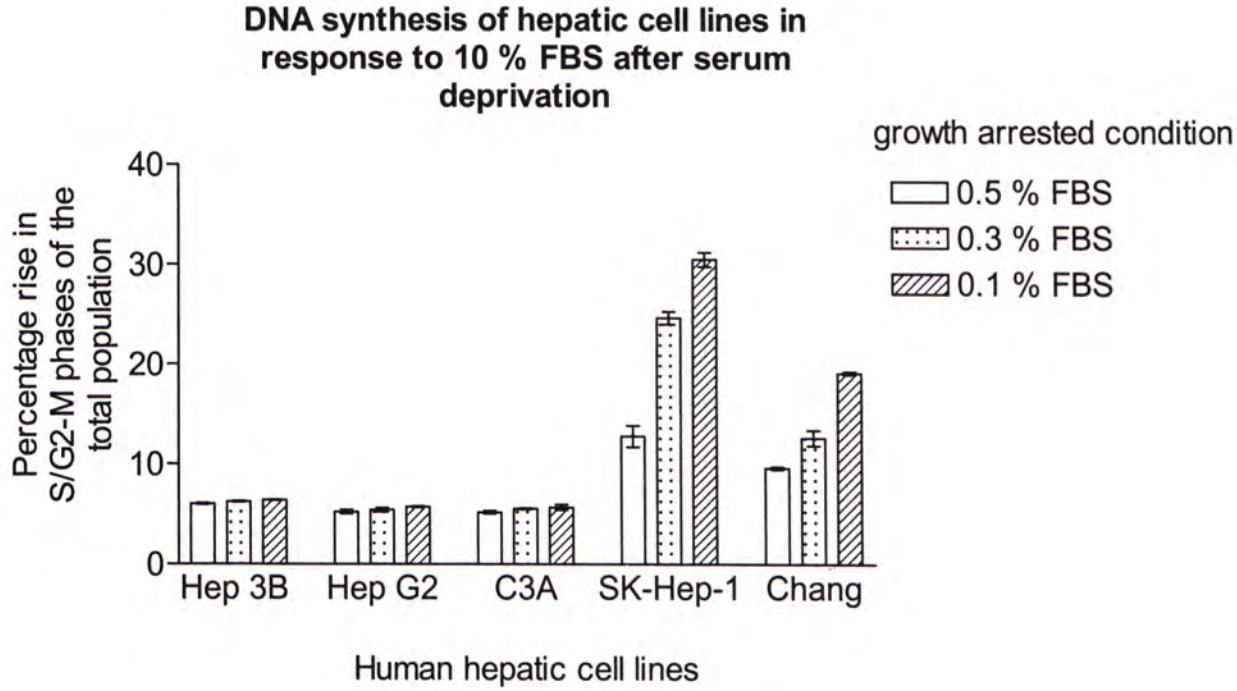



Figure 2. DNA synthesis of hepatic cell lines in response to 10 % FBS after serum deprivation. After serum deprivation for 72 hr with 0.5, 0.3, and 0.1 % FBS, Hep 3B, Hep G2, C3A, SK-Hep-1 and Chang cells, cells were incubated with 10 % FBS mitogenic stimulation for 21hr. Cell cycle analysis was performed after 21 hr. "Percentage rise in S/G2-M phases of the total population" was determined by the percentage of cells in S/G2-M phases with 10 % FBS stimulation minus the percentage of control cells in S/G2-M phases without stimulation. Data were mean of two experiments in duplicates $\pm$ S.D.

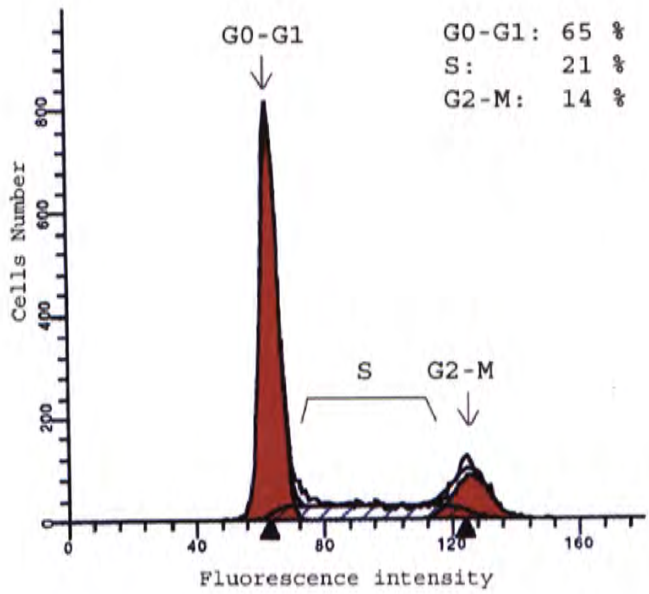
## 3.2 Detection of mitogenic activity of human bone marrow stromal cells on the selected cell line, SK-Hep-1 cells

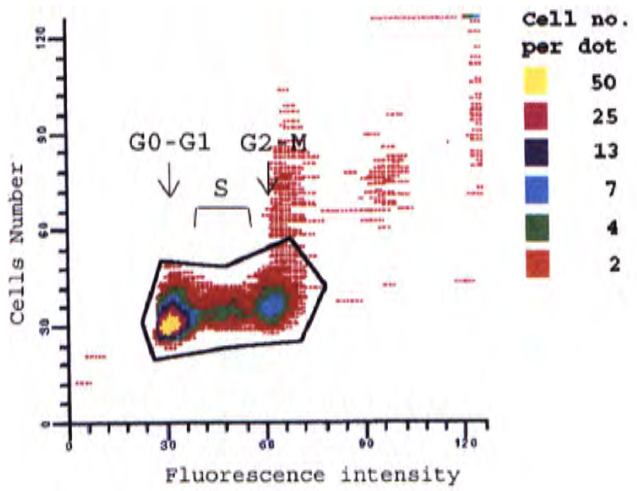
### 3.2.1 Cell cycle distribution of partially growth arrested SK-Hep-1 cells in response to mitogens

The mitogenic effect of human bone marrow stromal cellular extract on partially growth arrested SK-Hep-1 cells was determined by cell cycle analysis. The percentage of SK-Hep-1 cells in S/G2-M phases under the stimulation of FBS (Figure 3B) and stromal cellular extract of patient 1 (Figure 3C) were 30 % (S.D. = $\pm$ 0.2; n = 5) and 17 % (S.D. = $\pm$ 0.7; n = 5) increased, respectively as compared to the untreated control (Figure 3A). The mitogenic activity was defined by cell cycle distribution changes of SK-Hep-1 cells before and after mitogens incubation throughout the experiment.

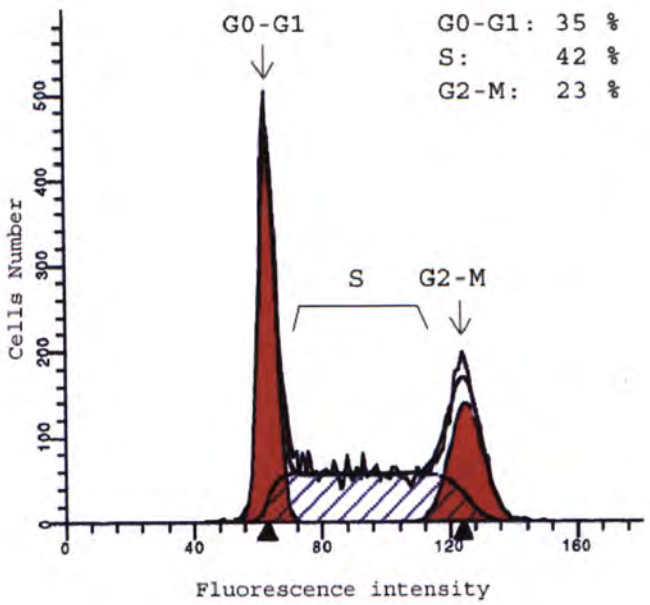
Figure 3. Cell cycle distribution of partially growth arrested SK-Hep-1 cells in response to mitogens. The representative diagrams of cell cycle distribution of partially growth arrested SK-Hep-1 cells without treatment (A), cells incubated with 10 % FBS for 21 hr (B) and cell incubated with 30 μg/ml bone marrow stromal cellular extract of patient 1 for 21 hr (C) were shown. The fluorescence intensity of cell stained with propidium iodide was measured by the flow cytometry. Those SK-Hep-1 cells that had an unreplicated complement of DNA, 2N were in G0-G1 phases. Those SK-Hep-1 cells that had a fully replicated complement of DNA, 4N were in G2-M phases. Those had an intermediate amount of DNA were in S phases. Diagrams were representative of five experiments in duplicates.

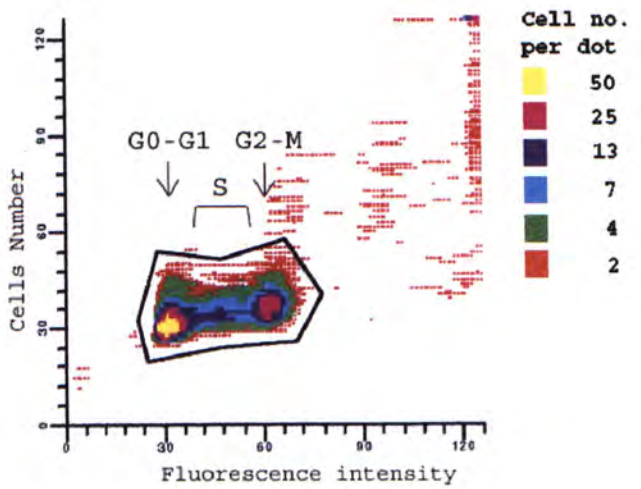
**A**


G0-G1
G0-G1: 65 %
S: 21 %
G2-M: 14 %
Cells Number
S
G2-M
Fluorescence intensity

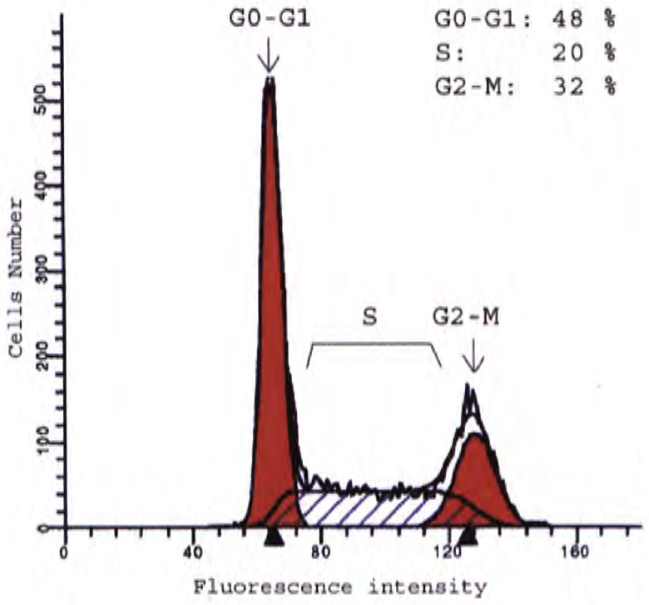

Cell no. per dot
50
25
13
7
4
2
G0-G1
S
G2-M
Cells Number
Fluorescence intensity

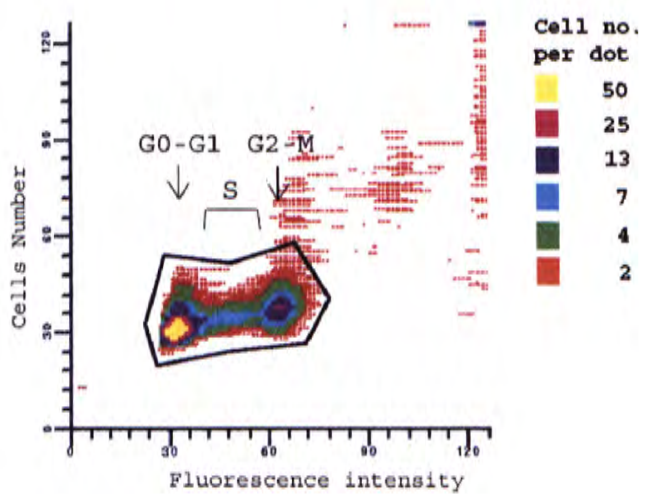
**B**


G0-G1
G0-G1: 35 %
S: 42 %
G2-M: 23 %
S
G2-M
Cells Number
0
100
200
300
400
500
0
40
80
120
160
Fluorescence intensity


G0-G1
S
G2-M
Cells Number
0
30
60
90
120
Fluorescence intensity
Cell no. per dot
50
25
13
7
4
2

C


G0-G1
G0-G1: 48 %
S: 20 %
G2-M: 32 %
S
G2-M
Cells Number
0
100
200
300
400
500
0
40
80
120
160
Fluorescence intensity


G0-G1
S
G2-M
Cells Number
0
30
60
90
120
Fluorescence intensity
Cell no. per dot
50
25
13
7
4
2

### 3.2.2 Time course on DNA synthesis of partially growth arrested SK-Hep-1 cells in response to FBS and bone marrow stromal cellular extract

The optimum mitogenic response of partially growth arrested SK-Hep-1 cells was determined through a time course experiment on DNA synthesis of SK-Hep-1 cells in response to FBS and stromal cellular extract of patient 1. The mitogenic activity was detectable by 12 hr and appeared to be maximal at about 21 hr (Figure 4). Similar result was found in cells under stimulation with stromal cellular extract of patient 1. Thus, the peak of mitogenic activity in partially quiescent SK-Hep-1 cells occurred at about 21 hr after mitogen inoculation and this inoculation time was used throughout the experiment.

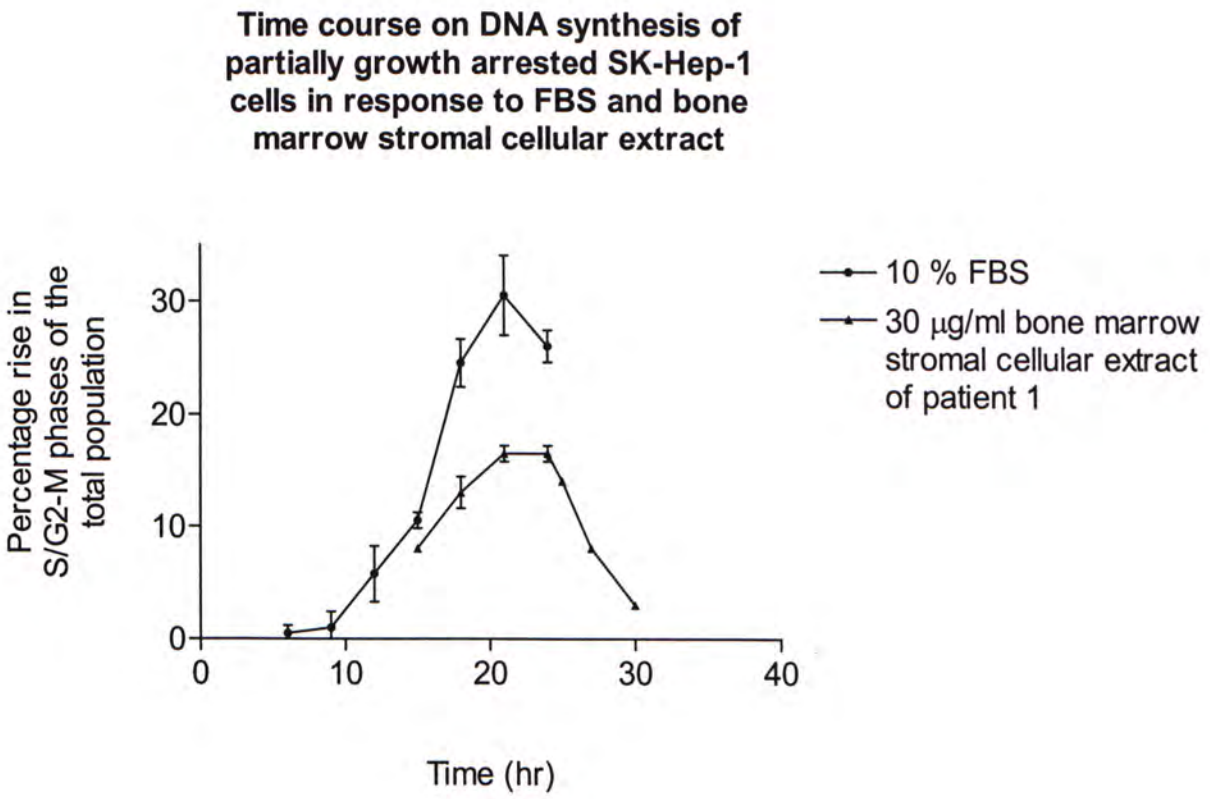



Figure 4. Time course on DNA synthesis of partially growth arrested SK-Hep-1 cells in response to FBS and bone marrow stromal cellular extract. Partially growth arrested SK-Hep-1 cells were incubated with 10 % FBS and 30 μg/ml stromal cellular extract of patient 1 for 6, 9, 12, 15, 18, 21, 24 hr and 15, 18, 21, 24, 25, 27, 30 hr, respectively. The mitogenic activity of 10 % FBS (1, -•-) and 30 μg/ml bone marrow stromal cellular extract of patient 1 (2, -▲-) on DNA synthesis of SK-Hep-1 cells from 6 to 30 hr were shown. "Percentage rise in S/G2-M phases of the total population" was determined by the percentage of cells in S/G2-M phases with mitogenic stimulation minus the percentage of control cells in S/G2-M phases without stimulation. Data were the mean of two experiments in duplicates $\pm$ S.D.

### 3.2.3 Dose response on DNA synthesis of partially growth arrested SK-Hep-1 cells in response to bone marrow stromal cellular extracts

Mitogenic activity of human bone marrow stromal cellular extract on partially growth arrested SK-Hep-1 cells was determined through a dose response experiment on DNA synthesis in response to stromal cellular extracts originated from four different patients. Dosage curve on DNA synthesis of SK-Hep-1 cells was observed upon stimulation with four different bone marrow stromal cellular extracts. The maximum percentage of cells increase in S/G2-M phases was 19 % under 30 μg/ml stromal cellular extract stimulation.

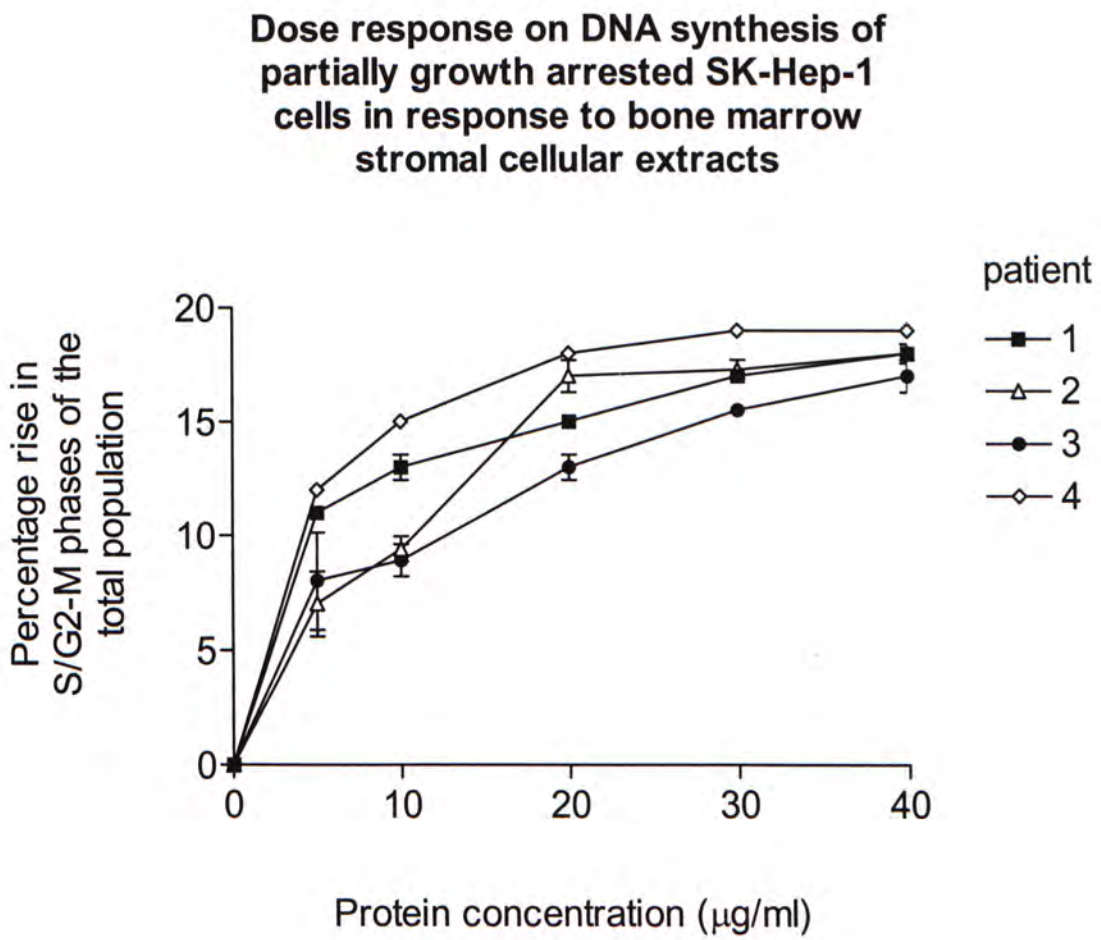


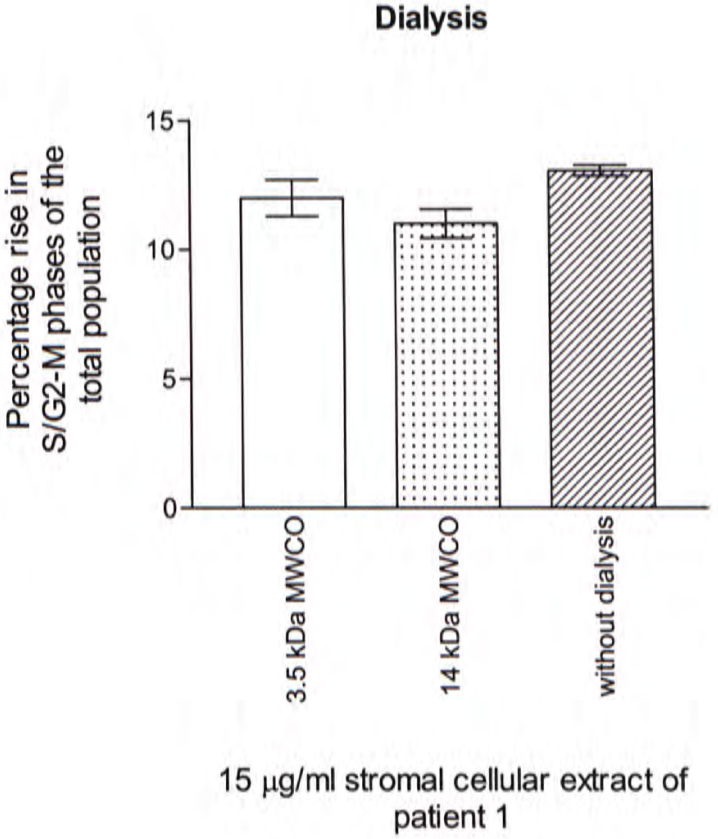

Figure 5. Dose response on DNA synthesis of partially growth arrested SK-Hep-1 cells in response to bone marrow stromal cellular extracts. The graph showed the mitogenic activities of 5, 10, 20, 30 and 40 μg/ml stromal cellular extracts originated from four different patients (1, -■-; 2, -Δ-; 3, -●-; 4, -◇-) on partially growth arrested SK-Hep-1 cells incubating for 21 hr. "Percentage rise in S/G2-M phases of the total population" was determined by the percentage of cells in S/G2-M phases with mitogenic stimulation minus the percentage of control cells in S/G2-M phases without mitogenic stimulation. Data were the mean of two experiments in duplicates ± S.D.

## 3.3 Characterization of hepatocyte mitogenic activity of bone marrow stromal cellular extract

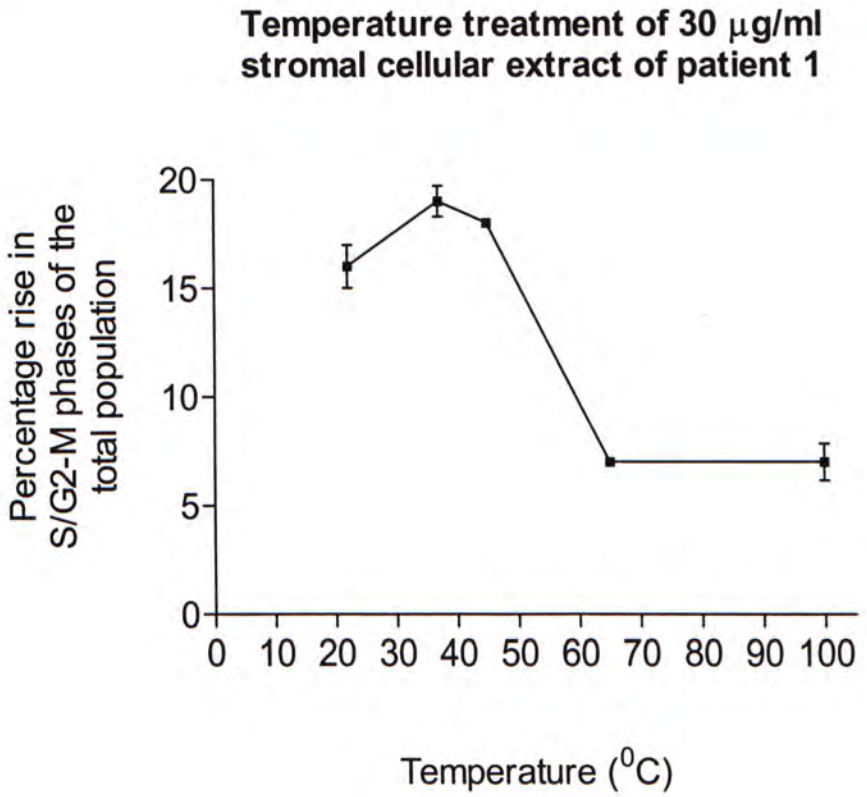
The physical properties of hepatocyte mitogenic activity of bone marrow stromal cellular extract including the approximate size, thermostability and resistance to proteinase digestion were characterized by dialysis, temperature treatment and proteolysis, respectively. The mitogenic activity of stromal cellular extract of patient 1 was about the same before and after dialyzed against 3.5 and 14 kDa MWCO dialysis tubing (Figure 6A). The mitogenic activity of stromal cellular extract of patient 1 lost progressively with 63 % reduction in mitogenic activity after $65^0$C heat treatment (Figure 6B). The mitogenic activity of stromal cellular extract of patient 1 lost after digested with trypsin or endoproteinase Lys-C with 88 % reduction and 76 % reduction in mitogenic activity after 37 $^0$C digestion for 30 min (Figure 6C). These results suggested that most of the hepatic mitogenic effect of stromal cellular extract from patient 1 was heat labile proteins with size larger than 14 kDa.

Figure 6. Characterization of hepatocyte mitogenic activity of bone marrow stromal cellular extract. The data showed the mitogenic activities of stromal cellular extract of patient 1 on partially growth arrested SK-Hep-1 cells pretreated with 3.5 and 14 kDa MWCO dialysis at 4 $^{0}$C overnight (A); 22, 37, 45, 65 and 100 $^{0}$C temperature treatment for 30 min (B); and 0.3 μg/ml trypsin and 0.3 μg/ml endoproteinase Lys-C proteolysis at 37$^{0}$C for 30 min (C). "Percentage rise in S/G2-M phases of the total population" was determined by the percentage of cells in S/G2-M phases with mitogenic stimulation minus the percentage of control cells in S/G2-M phases without stimulation. Data were the mean of two experiments in duplicates $\pm$ S.D.

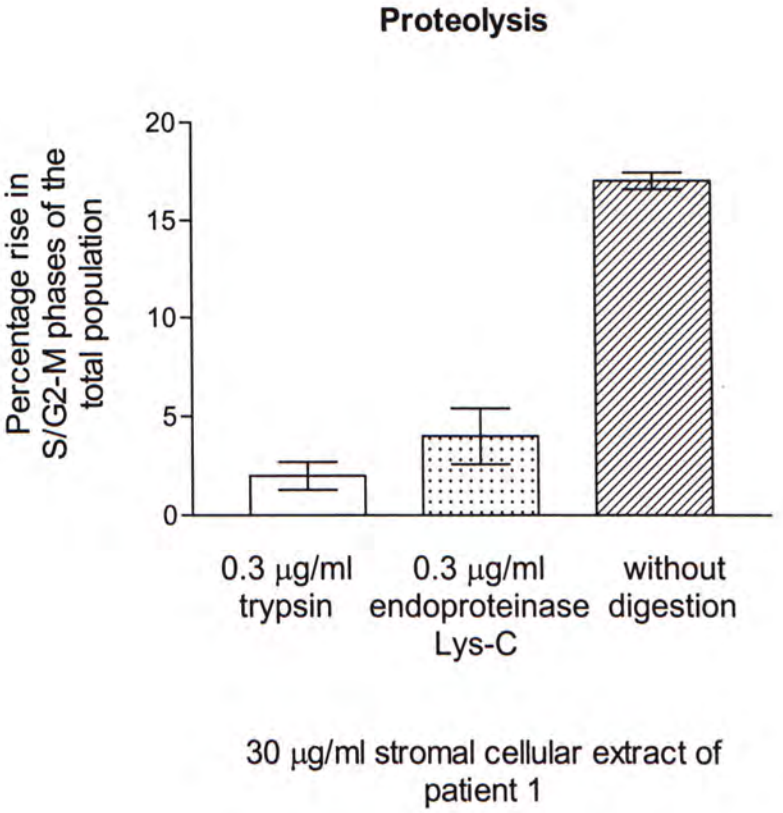
**A**


Dialysis
Percentage rise in S/G2-M phases of the total population
15
10
5
0
3.5 kDa MWCO
14 kDa MWCO
without dialysis
15 µg/ml stromal cellular extract of patient 1

**B**


Temperature treatment of 30 μg/ml stromal cellular extract of patient 1
Percentage rise in S/G2-M phases of the total population
20
15
10
5
0
0 10 20 30 40 50 60 70 80 90 100
Temperature (0C)

C


Proteolysis
Percentage rise in S/G2-M phases of the total population
20
15
10
5
0
0.3 µg/ml trypsin
0.3 µg/ml endoproteinase Lys-C
without digestion
30 µg/ml stromal cellular extract of patient 1

## 3.4 Performing a preliminary test on the difference between bone marrow stromal cellular extract and other growth factors

### 3.4.1 Mitogenic response of SK-Hep-1 cells in response to bone marrow stromal cellular extract and other growth factors

The mitogenic response of partially growth arrested SK-Hep-1 cells in response to stromal cellular extract of patient 1 and other known hepatocyte stimulatory growth factor, namely HGF, EGF, TGF-α and insulin were determined. The percentages of cells increase in S/G2-M phases were 12, 7, 5, 10 and 18 % in 30 ng/ml HGF, 1 ng/ml EGF, 1 ng/ml TGF-α, 2 μg/ml insulin and 30 μg/ml stromal cellular extract of patient 1, respectively.

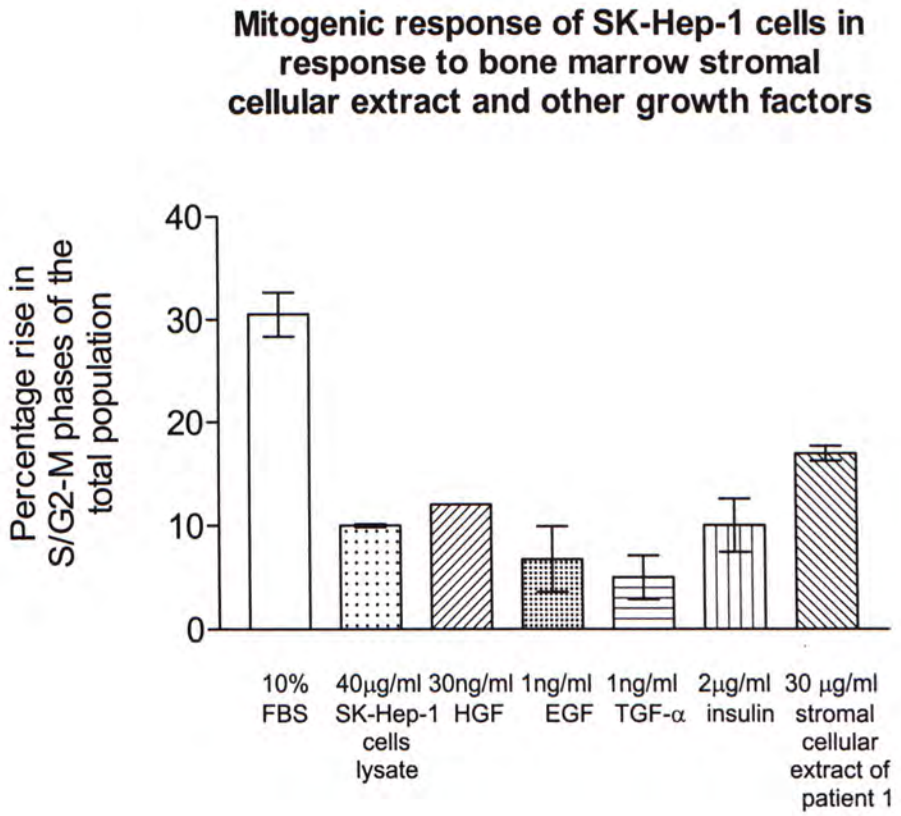
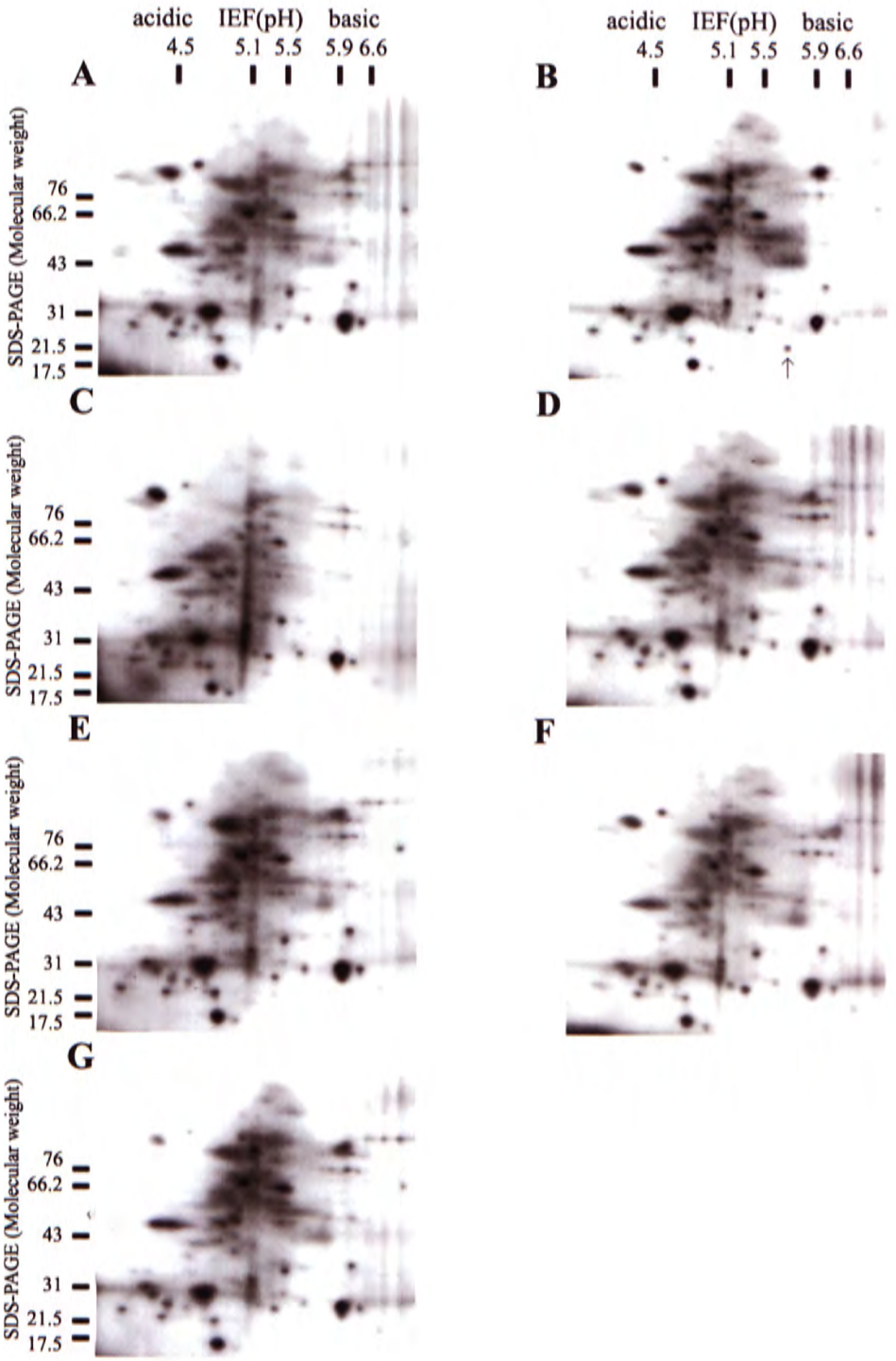



Figure 7. Mitogenic response of SK-Hep-1 cells in response to bone marrow stromal cellular extract and other growth factors. The chart showed the mitogenic activities of 10 % FBS, 40 μg/ml SK-Hep-1 cells lysate, 30 ng/ml HGF, 1 ng/ml EGF, 1 ng/ml TGF-α, 2μg/ml insulin and 30 μg/ml stromal cellular extract of patient 1 on partially growth arrested SK-Hep-1 cells after incubation for 21 hr. "Percentage rise in S/G2-M phases of the total population" was determined by the percentage of cells in S/G2-M phases with mitogenic stimulation minus the percentage of control cells in S/G2-M phases without stimulation. Data were the mean of two experiments in duplicates ± S.D.

### 3.4.2 Early intracellular signaling of SK-Hep-1 cells in response to bone marrow stromal cellular extract and other growth factors

The early intracellular signaling of SK-Hep-1 cells in response to bone marrow stromal cellular extract of patient 1 was compared with other known hepatocyte stimulatory growth factor, namely HGF, EGF, TGF-α and insulin. These results showed that the general phorphorylation pattern of partially growth arrested SK-Hep-1 cells in response to either stromal cellular extract of patient 1 or stimulatory growth factors were as same as the untreated control for 15, 30, 45 and 60 min except under stimulation with 30 μg/ml stromal cellular extract of patient 1 for 30 min. A unique phosphoprotein spot was detected with molecular weight at 21.5 kDa and pI at 5.7 (Figure 6B).

Figure 8. Early intracellular signaling of SK-Hep-1 cells in response to bone marrow stromal cellular extract and other growth factors. These autoradiographs showed the general phorphorylation pattern of growth arrested SK-Hep-1 cells in response to stromal cellular extract of patient 1 and other growth factors stimulation. The general phosphorylation pattern of growth arrested SK-Hep-1 cells without treatment (A), cells incubated with 30 μg/ml stromal cellular extract of patient 1 for 30 min (B), cells incubated with 10 % FBS for 30 min (C), cells incubated with 30 ng/ml HGF for 30 min (D), cells incubated with 2 μg/ml insulin for 30 min (E), cells incubated with 1 ng/ml EGF for 30 min (F) and cells incubated with 1 ng/ml TGFα for 30 min (G). Results shown were representation of triplicate.


acidic
IEF(pH)
basic
4.5
5.1
5.5
5.9
6.6
A
B
C
D
E
F
G
SDS-PAGE (Molecular weight)
76
66.2
43
31
21.5
17.5

# Chapter 4 Discussion

## 4.1 Selection of human hepatic cell line for the detection of mitogenic activity

Among hepatic cell lines, Hep 3B, Hep G2, C3A, SK-Hep-1 and Chang cells, it was demonstrated that SK-Hep-1 and Chang cells were enriched at G0-G1 phases after serum deprivation. And these growth arrested hepatic cells were able to continue through S/G2-M phases of the cell cycle upon 10 % FBS stimulation without death of the cells. This result suggested that both SK-Hep-1 and Chang cells can be growth arrested. Since SK-Hep-1 cells showed a higher percentage changes, about one-third higher upon FBS stimulation. Thus SK-Hep-1 cells were used throughout the experiment in order to maximize the sensitivity for detection of mitogenic activity. And the concentration of FBS being used to deprive cells was 0.1 % for 72 hr. One of the limitation of using SK-Hep-1 cells was that the onwards data obtained might only unique to SK-Hep-1 cells or *in vitro* condition.

Serum withdrawal requires extensive optimization of both amount of serum withdrawn and the length of withdrawal. The concentration of 0.5 % - 0.1 % FBS covered the commonly used percentage of FBS for serum deprivation (Campisi et al., 1984). Although the increase in length of withdrawal might increase the percentage of Hep 3B, Hep G2, C3A cells synchronization at G0-G1 phases, the death of the cells will increase at the same time. Serum withdrawn for 72 hr was the commonly used time in the balance between the percentage of cells being synchronized and the number of death of cells (Campisi et al., 1984).

## 4.2 Mitogenic activity of human bone marrow stromal cells on the selected cell line, SK-Hep-1 cells

In this study, it was demonstrated that a dosage dependent hepatocyte mitogenic activity of human bone marrow stromal cellular extracts originated from four different patients. This result suggested that human bone marrow stromal cells had specific hepatocyte mitogenic activity. The finding was in agreement with the report of Michalopoulos and DeFrances who reviewed the stimulating effect of stromal-derived HGF on DNA synthesis of hepatocytes and the essential role of stromal-derived IL-6 in early signaling pathway of hepatocyte proliferation (Michalopoulos and DeFrances, 1997). Thus, bone marrow stromal-derived cytokines, HGF and IL-6 were potentially responsible for the stimulating effect on DNA synthesis of SK-Hep-1 cells in this *in vitro* experiment.

Liver is the principal hematopoietic organ in the human fetus from the $6^{th}$ through the $22^{nd}$ gestational weeks. Different hematopoietic lineages including myelopoietic, erythropoietic and lymphopoietic lineages proliferate and differentiate in the fetal liver from stem cells that migrate from the yolk sac (Timens and Kamps, 1997). Fetal hepatocytes extracellular matrix contains cytokines necessary for growth and development of hematopoietic cells in addition to itself. Those bone marrow stromal-derived cytokines involved in hepatocytes proliferation namely HGF, IL-6 and TGF-β are also necessary in the myelopoiesis particularly in the CFU-GM lineage (Bot et al., 1989; Cashman et al., 1990; Kmiecik et al., 1992; Mizuno et al., 1993; Galimi et al., 1994; Nishino et al., 1995; Liu et al., 1997; Takai et al., 1997). CD15 is a distinctive cell surface marker representing the colony formation from CFU-GM. Interestingly, in $20^{th}$ gestational week of fetal liver, the myelopoietic

CD15 positive cells were localized mainly in periportal zone (Timens and Kamps, 1997), the first portion of hepatocytes in hepatic lobules undergoing proliferation after PHx. The reason of such topographic distribution of myelopoietic cells remains unknown. In addition to the evidence that hepatocytes can arise from cell populations originating in bone marrow (Petersen et al., 1999), those potent bone marrow stromal-derived cytokines involved specifically in CFU-GM lineage development, namely G-CSF, GM-CSF and M-CSF (Nicola, 1987) may also be responsible for the stimulating effect on DNA synthesis of SK-Hep-1 cells in this *in vitro* experiment.

## 4.3 Characterization of hepatocyte mitogenic activity of bone marrow stromal cellular extract

Most of the stimulating effect of bone marrow stromal cellular extract of patient 1 on DNA synthesis of SK-Hep-1 cells lost after proteolysis and heat treatment suggested that the major stromal-derived hepatocyte mitogens were heat labile proteins. And the stimulating effect of bone marrow stromal cellular extract of patient 1 on DNA synthesis of SK-Hep-1 cells was maintained after dialysis indicated that the stromal-derived hepatocyte mitogens were not a small molecular. These findings were in agreement with the physical properties of all above potential stromal-derived hepatocyte cytokines being suggested. As all HGF, IL-6, G-CSF, GM-CSF and M-CSF are heat labile proteins with molecular weight, 85, 25, 25, 23 and 70 kDa, respectively.

Interestingly, some of the stimulating effect of bone marrow stromal cellular extract of patient 1 on DNA synthesis of SK-Hep-1 cells can not be eliminated after

proteolysis and heat treatment. This might be due to the present of non-protein nature mitogens or heat - stable proteins that resisted towards digestion with trypsin and endoproteinase Lys-C in the stromal cellular extract. Such observation had also been reported in human placental extract (Liu et al., 1998) that human placental extract contains a heat-stable fraction, which can stimulate the growth of hepatocytes.

## 4.4 Performing a preliminary test on the difference between bone marrow stromal cellular extract and other growth factors

The infusion of HGF, EGF or TGF-α in normal rat directly through the portal vein could only have a little effect on hepatic DNA synthesis. It indicated that either multiple cytokines or an unknown cytokine was required for the initiation of hepatocytes proliferation after PHx (Webber et al., 1994). In this study, given together, no data can support whether the hepatic mitogenic activity of stromal cells was triggered by a known cytokine, an unknown cytokine or multiple stromal-derived cytokines. The effect of stromal cellular extract on early intracellular signaling of SK-Hep-1 cells was performed with a parallel control on its DNA synthesis to examine the potential of difference between existing growth factors and stromal cellular extract.

A different phosphoprotein was detected in SK-Hep-1 cells at 30 min after inoculation with bone marrow stromal cellular extract. This phosphorylated protein was undetectable in SK-Hep-1 cells when inoculated with HGF, EGF, TGF-α, insulin or FBS alone. This showed that a different intracellular signaling messenger was being up regulated in SK-Hep-1 cells as the result of stromal cellular extract

treatment. The parallel experiment on increasing DNA synthesis of SK-Hep-1 cells was obtained. However, no data can further correlate this phosphorylated signal and the DNA synthesis, and whether this signal was a result from a known cytokine, an unknown cytokine or multiple stromal-derived cytokines. This experiment provided evidence leading to detail study.

Interestingly, the mitogenic activity of FBS was always higher than the other mitogens or stromal cellular extracts throughout the study. This reflected the fact that FBS has tremendous amount of different cytokines. And the initiation of proliferation of cells always involve in many different cytokines.

Hepatocytes exit from their normally quiescent state within 30 min after PHx as the immediate-early genes are rapidly induced (Taub, 1996). This expression is independent of protein synthesis and appears to result from mitogenic stimuli. Many of these immediate-early genes contain in their promotor region sequences reactive to the transcription factor, nuclear factor kappa B (NF-κB) and STAT3. Thus, activation of NF-κB and STAT3 are likely to be a major part of the intracellular signal transduction pathways leading to mitosis. The activation of NF-κB occurs within a minute after PHx (Cressman et al., 1994) and the activation of STAT3 occurs within 30 min peaking at 3 hr (Cressman et al., 1995). The different intracellular signaling was detected in SK-Hep-1 cells by 30 min after inoculation with bone marrow stromal cellular extract suggesting this signaling messenger may involve in early intracellular signaling of SK-Hep-1 cells proliferation.

Coupling between HGF and its transmembrane bound tyrosine kinase receptor, c-Met activates the tyrosine Y1349 and Y1356 activity on its intercellular domain

resulting in mitogenic response in a wide variety of cells including hepatocytes (Ponzetto et al., 1994), while serine 985 in c-Met is the major phosphorylation site for protein kinase C or other $Ca^{2+}$-dependent kinase(s) to down regulation this mitogenic response (Gandino et al., 1994). To detect this phosphorylated c-Met protein, purification from cells lysate through immunoprecipitation with monoclonal, c-Met/anti-c-Met conjugated with protein A-Sepharose should be carried out before gel loading. The unique intracellular signaling messenger detected in SK-Hep-1 cells was resolved on 2D gel without antibody purification such as affinity chromatography or immunoprecipitation for a specific protein. One would expect that this strong phosphoprotein signal detected might involve in an amplifying cascade.

## 4.5 Possible directions for future investigation

Human bone marrow stromal cells secrete a lot of cytokines and some of these cytokines are already shown to be essential in triggering hepatocytes proliferation after liver regeneration. To examine the potential of difference between stromal-derived factors and other growth factors, partially growth arrested SK-Hep-1 cells can be incubated with both stromal cellular extract and other growth factors or FBS simultaneously to determine whether there is a synergistic effect. If a synergistic effect obtained, this indirect evidence can suggest that a difference might occur between stromal-derived factor and other cytokines on stimulation of hepatocytes proliferation.

To screen for the stromal-derived factors that was responsible for the stimulation of hepatocytes, the direct method is to purify the factors by protein chromatography

such as ion-exchange chromatography and gel filtration. The mitogenic activity of different fractions obtained from separated stromal-derived extracts could be examined by partially growth arrested SK-Hep-1 cells. In order to avoid cell type-specified result, primary hepatocytes culture should be used for investigation in addition to SK-Hep-1 cells.

## 4.6 Conclusions

(i) human bone marrow stromal cells had mitogenic activity on SK-Hep-1 cells; and

(ii) these stromal-derived hepatocyte mitogens were mainly proteins in nature.

# Chapter 5 Appendices

## 5.1 Reagents and solutions

Use Milli-Q water (Millipore Corporation, Bedford, MA, USA, ZMQS5VF0Y) for all the preparation of buffers.

### 5.1.1 Selection of human hepatic cell line for the detection of mitogenic activity

| Equipment or reagent | Supplier | Code no. |
|---|---|---|
| 0.22 μm filter | USA Scientific, Inc. | USA F192 |
| 35 μm stainer cap | Falcon, Becton Dickinson | 2235 |
| C3A cells | American Type Culture Collection | CRL-10741 |
| Chang cells | American Type Culture Collection | CCL-13 |
| $CO_2$ water jacketed incubator | Forma Scientific Inc. | 3111 |
| Coulter Particle Count and Size Analyser | Coulter electronics Ltd. | Z2 |
| Flow cytometry | Becton Dickinson | FASC Vantage |
| Hep 3B cells | American Type Culture Collection | HB-8064 |
| Hep G2 cells | American Type Culture Collection | HB-8065 |
| Microcentrifuge | Eppendorf | 5417R |
| Penicillin and streptomycin | Sigma | P0781 |
| SK-Hep-1 cells | American Type Culture Collection | HTB-52 |

| Cell fixation solution | Σ 500ml | working concentration |
|---|---|---|
| • Absolute ethanol (Riedel-de Haën, 32221, FW46.07) | 475 ml | 95 % (V/V) |
| • 1 X PBS | 25 ml | 5 % (V/V) |

Store at 4 $^0$C.

| Dulbecco's modified Eagle's medium | Σ 2 L | working concentration |
|---|---|---|
| • DMEM (GIBCO, 12800-017) | 2 pack | N/A |
| • Sodium bicarbonate ($NaHCO_3$) (Sigma, S2127, FW106.0) | 7.4 g | 0.37 % (W/V) |

Adjust pH to 7.50, 0.22 μm filter and store at 4 $^0$C.

| Froze cells medium | Σ 10 ml | working concentration |
|---|---|---|
| • Culture medium (GIBCO) | 6.7 ml | 67 % (V/V) |
| • Fetal bovine serum (FBS) (GIBCO, 26140-079) | 2.5 ml | 25 % (V/V) |
| • Dimethyl sulphoxide (DMSO) (Sigma, D2650) | 0.8 ml | 8 % (V/V) |

Freshly prepared before use.

| Minimum essential medium | Σ 2 L | working concentration |
|---|---|---|
| • MEM (GIBCO, 61100-061) | 2 pack | N/A |
| • Sodium bicarbonate ($NaHCO_3$) (Sigma, S2127, FW106.0) | 4.4 g | 0.22 % (W/V) |

Adjust pH to 7.50, 0.22 μm filter and store at 4 $^0$C.

| 10 X Phosphate-buffered saline (PBS): | Σ 2L | working concentration |
|---|---|---|
| • Sodium Chloride (NaCl) (Sigma, S9888, FW 58.44) | 160 g | 137 mM |
| • Potassium chloride (KCl) (Sigma, P4504, FW 74.55) | 4 g | 2.7 mM |
| • Sodium Phosphate, dibasic, anhydrous ($Na_2HPO_4$) (Sigma, S0876, FW 141.96) | 12.2g | 4.3 mM |
| • Potassium Phosphate, monobasic, anhydrous ($KH_2PO_4$) (Sigma, P5379, FW 136.1) | 4 g | 1.4 mM |

Adjust pH to 7.4 and store at room temperature.

| Propidium iodide stock solution | Σ 10 ml | working concentration |
|---|---|---|
| • Propidium iodide (Sigma, P4170, FW668.4) | 25 mg | 50 μg/ml |
| • 1 X PBS | 10 ml | N/A |

Aliquot to 400 μl/vial and store at –20 $^0$C in dark.

| Ribonuclease A stock solution | Σ 10ml | working concentration |
|---|---|---|
| • Ribonuclease A (RNase A) (Sigma, R4875, MW13,700) | 1 g | 0.1 % (W/V) |
| • 1 X PBS | 10 ml | N/A |

Aliquot to 200 µl/vial and store at –20 $^{0}$C.

### 5.1.2 Detection of mitogenic activity of human bone marrow stromal cells on the selected cell line, SK-Hep-1 cells

| Equipment or reagent | Supplier | Code no. |
|---|---|---|
| Spectrophotometer | Beckman | DU650 |
| Ultracentrifuge | Beckman Instruments, Inc. | XL-70 |
| Ultrasonic Processor | Cole-Parmer Instrument Co. | torbeo 36800 |

| Bovine serum albumin standard stock solution | Σ 10 ml | Final concentration |
|---|---|---|
| • Bovine serum albumin (BSA) (Sigma, A2153) | 20 mg | 2 mg/ml |

Aliquot to 50 µl/vial and store at –20 $^{0}$C.

Pipette 30 µl bovine serum albumin standard stock solution to 270 µl Milli-Q before use.

| RPMI medium 1640 | Σ 2 L | working concentration |
|---|---|---|
| • RPMI medium 1640 (GIBCO, 23400-021) | 1 pack | N/A |
| • Sodium bicarbonate ($NaHCO_3$) (Sigma, S2127, FW106.0) | 4 g | 0.2 % (W/V) |

Adjust pH to 7.50, 0.22 µm filter and store at 4 $^{0}$C.

Standard curve

Prepare the standard curve and measure the sample as following table:

| Concentration (μg/ml) | $H_2O$ (μl) | BSA STD / sample (μl) | Solvent used in sample (μl) | Dye Reagent Concentrate (Bio-Rad, 500-0006) (μl) |
|---|---|---|---|---|
| 0 | 790 | 0 | 10 | 200 |
| 5 | 765 | 25 | 10 | 200 |
| 10 | 740 | 50 | 10 | 200 |
| 15 | 715 | 75 | 10 | 200 |
| 20 | 690 | 100 | 10 | 200 |
| Sample | 790 | 10 | / | 200 |

### 5.1.3 Characterization of hepatocyte mitogenic activity of bone marrow stromal cellular extract

| Equipment or reagent | Supplier | Code no. |
|---|---|---|
| Dialysis tubings MWCO 3.5 kDa | Spectrum Laboratories Inc. | 132720 |
| Dialysis tubings MWCO 14 kDa | Spectrum Laboratories Inc. | 132676 |
| Endoproteinase Lys-C | Roche | 1047825 |
| TLCK | Roche | 874493 |
| Trypsin | Roche | 1418025 |

| 0.1 M EDTA solution | Σ 2 L | working concentration |
|---|---|---|
| • 0.5 M EDTA stock solution | 400 ml | 0.1 M |

Store at room temperature.

| 0.5 M EDTA stock solution | Σ 500 ml | Working concentration |
|---|---|---|
| • Ethylenediaminetetraacetic acid (EDTA) (Sigma, E6511, FW380.2) | 95.05 g | 0.5 M |

Store at room temperature.

| Sodium bicarbonate / EDTA solution | Σ 2 L | working concentration |
|---|---|---|
| • 0.5 M EDTA stock solution | 4 ml | 1 mM |
| • Sodium bicarbonate ($NaHCO_3$) (Sigma, S2127, FW106.0) | 40 g | 2 % |

Store at room temperature.

### 5.1.4 Performing a preliminary test on the difference between bone marrow stromal cellular extract and other growth factors

| Equipment or reagent | Supplier | Code no. |
|---|---|---|
| $^{32}P_i$, 0.1 mCi/ml, carrier free | Pharmacia | PBS11-10mCi |
| Automated Gel Stainer | Pharmacia | 80-6395-02 |
| BioMax MR autoradiography film | Eastman Kodak Company | 8912560 |
| EN$^3$HANCE | NEN™ | NEF981 |
| ExcelGel SDS | Pharmacia | 80-1255-53 |
| Gel Dryer | Bio-Rad | 583 |
| Immobiline DryStrip Kit | Pharmacia | 18-1004-30 |
| Immobiline DryStrip Reswelling Tray | Pharmacia | 80-6371-84 |
| IPG Cover Fluid | Pharmacia | 17-1335-01 |
| IPG Strip, pH 3 – 10 linear, 11 cm | Pharmacia | 18-1016-61 |
| Labeling medium, phosphate free DMEM | GIBCO-BRL Life Technologies | 11971-025 |
| Multi Temp III Thermostatic Circulator | Pharmacia | 18-1102-78 |
| Multiphor II unit | Pharmacia | 18-1018-06 |
| PlusOne Silver Staining Kit, Protein | Pharmacia | 17-1150-01 |
| Power supply | Pharmacia | EPS 3501 XL |
| Two-dimensional SDS-PAGE standards | Bio-Rad | 161-0320 |

| Developing solution | Σ 1250 ml | working concentration |
|---|---|---|
| • Sodium carbonate (Pharmacia, 17-1150-01, FW106.0) | 31.25 g | 2.5 % (W/V) |

Store at room temperature.

Add 35 µl 37 % formaldehyde (Pharmacia, 17-1150-01) to 175 ml developing solution before use.

| Epidermal growth factor stock solution | 1000 µl | Final concentration |
|---|---|---|
| • EGF (Calbiochem, 324831) | 200 µg | 200 ng/µl |
| • 1X PBS | 1000 µl | N/A |

Aliquot to 10 µl/vial and store at –80 $^{0}$C; 100 X dilution to 2 ng/µl before use.

| Fixer solution | Σ 1 L | working concentration |
|---|---|---|
| • Glacial acetic acid (Riedel-de Haën, 27225, FW60.05) | 100 ml | 10 % (V/V) |
| • Absolute ethanol (Riedel-de Haën, 32221, FW46.07) | 400 ml | 40 % (V/V) |

Store at room temperature.

| Hepatocyte growth factor stock solution | 500 µl | Final concentration |
|---|---|---|
| • HGF (Calbiochem, 375228) | 5 µg | 10 ng/µl |
| • 1 X PBS | 500 µl | N/A |

Aliquot to 30 µl/vial and store at –80 $^{0}$C.

| Insulin stock solution | 50 ml | Final concentration |
|---|---|---|
| • insulin (Calbiochem, 407694) | 50 mg | 1 mg/ml |
| • 1 X PBS | 50 ml | N/A |

Aliquot to 1 ml/vial and store at –80 $^{0}$C.

| Lysis solution: | Σ 40 ml | working concentration |
|---|---|---|
| • Urea (Sigma, U1250, FW60.06) | 19.2 g | 8 M |
| • Triton X-100 (Sigma, X-100) | 1.6 g | 4 % (W/V) |
| • Trizma base (Tris base) (Sigma, T1410, FW121.1) | 0.194 g | 40 mM |
| • $_{DL}$-Dithiothreitol ($_{DL}$-DTT) (Sigma, D0632, FW154.2) | 0.123 g | 20 mM |

Aliquot to 300 µl/vial and store at –20 $^{0}$C.

| Preserving solution | Σ 1 L | working concentration |
|---|---|---|
| • Glycerol (Sigma, G7757, FW92.09) | 87 ml | 8.7 % (V/V) |

Store at room temperature.

| Rehydration stock solution with IPG buffer: | Σ 25 ml | working concentration |
|---|---|---|
| • Urea (Sigma, U1250, FW60.06) | 12 g | 8 M |
| • Triton X-100 (Sigma, X-100) | 0.5 g | 2 % (W/V) |
| • IPG Buffer pH3-10L (Pharmacia, 17-6000-87) | 125 µl | 0.5 % |
| • Bromophenol blue (Sigma, B6131, FW691.9) | a few grains | trace |
| • $_{DL}$-Dithiothreitol ($_{DL}$-DTT) (Sigma, D0632, FW154.2) | 75 mg | 0.3 % |

Aliquot to 200 µl/vial and store at –20 $^{0}$C.

| SDS equilibration buffer with DTT / iodoacetamide | Σ 200 ml | working concentration |
|---|---|---|
| • 0.5 M Tris-HCl stock solution | 20 ml | 0.1 M |
| • Urea (Sigma, U1250, FW60.06) | 72 g | 6 M |
| • Glycerol (Sigma, G7757, FW92.09) | 60 ml | 30 % (V/V) |
| • Sodium dodecyl sulfate (SDS) (Sigma, L4509, FW288.4) | 2 g | 1 % (W/V) |
| • Bromophenol blue (Sigma, B6131, FW691.9) | a few grains | trace |
| iodoacetamide only | | |
| • $_{DL}$-Dithiothreitol ($_{DL}$-DTT) (Sigma, D0632, FW154.2) / | 1 g / | 0.5 % (W/V) / |
| Iodoacetamide (Sigma, I1149, FW185.0) | 9 g | 4.5 % (W/V) |

Aliquot to 10 ml/vial and store at –20 $^{0}$C.

| Sensitizing solution | Σ 1250 ml | working concentration |
|---|---|---|
| • Sodium acetate (Pharmacia, 17-1150-01, FW136.1) | 85 g | 6.8 % (W/V) |
| • 5 % sodium thiosulfate (Pharmacia, 17-1150-01, FW158.1) | 50 ml | 0.2 % (W/V) |
| • Absolute ethanol (Riedel-de Haën, 32221, FW46.07) | 375 ml | 30 % (V/V) |

Store at room temperature.

Add 0.875 ml 25 % glutardialdehyde (Pharmacia, 17-1150-01) to 175 ml sensitizing solution before use.

| Silver solution | Σ 1250 ml | working concentration |
|---|---|---|
| • 2.5 % silver nitrate solution (Pharmacia, 17-1150-01) | 125 ml | 0.25 % (W/V) |

Store in dark at room temperature.

Add 70 µl 37 % formaldehyde (Pharmacia, 17-1150-01, FW30.03) to 175 ml silver solution before use.

| Stop solution | Σ 1250 ml | working concentration |
|---|---|---|
| • EDTA – $Na_2$ (Pharmacia, 17-1150-01, FW380.2) | 18.25 g | 1.46 % (W/V) |

Store at room temperature.

| Transforming growth factor-α stock solution | 500 µl | Final concentration |
|---|---|---|
| • TGF-α (Calbiochem, 616430) | 100 µg | 200 ng/µl |
| • 1X PBS | 500 µl | N/A |

Aliquot to 10 µl/vial and store at –80 $^0$C; 100 X dilution to 2 ng/µl before use.

| 0.5 M Tris-HCL stock solution | Σ 500 ml | working concentration |
|---|---|---|
| • Trizma base (Tris base) (Sigma, T1410, FW121.1) | 30.5 g | 0.5 M |

First dissolve in 300 ml $H_2O$. Add 19 ml 37 % concentrated HCl and then adjust pH slowly with 1 N HCl to 6.8. Filter and store at 4 $^0$C.

| 20mM Tris-HCl stock solution: | Σ 1L | working concentration |
|---|---|---|
| • Trizma base (Tris base) (Sigma, T1410, FW121.1) | 24.22g | 20mM |

Adjust pH to 7.50 after 10 fold dilution and store at room temperature.

# Chapter 6 References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